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(57) Abstract

The present invention describes the isolation and purification of biologically and immunologically active histidine-tagged Shiga toxins (His-tagged), a toxin associated with HC and the potentially life-threatening sequela HUS transmitted by strains of pathogenic bacteria. The present invention describes how his-tagging greatly simplifies and expedites purifying Shiga toxins, and describes an improved method for such purification. One aspect of the invention is obtaining and using Shiga toxoids that are immunoreactive but not toxic. Another aspect of the invention is obtaining and using fusion proteins of His-tagged Shiga toxins or toxoids. Yet another aspect of the invention is obtaining and using antibodies to His-gagged Shiga toxins, toxoids, or Shiga toxin/toxoid fusion proteins.

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Description

HISTIDINE-TAGGED SHIGA TOXINS, TOXOIDS, AND PROTEIN FUSIONS WITH SUCH TOXINS AND TOXOIDS, METHODS FOR THE PURIFICATION AND PREPARATION THEREOF

GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed, and used for governmental purposes without payment of royalties to us thereon.

FIELD OF THE INVENTION

The invention relates to a family of multi-unit bacterial proteins that are associated with hemorrhagic colitis and the life-threatening sequela, hemolytic uremic syndrome. These proteins, defined as members of the "Shiga toxin family," have been tagged with histidine residues. The invention further relates to a non-toxinogenic but immunoreactive form of histidine-tagged Shiga toxins, or toxoids. Moreover, the invention relates to fusion proteins obtained by combining histidine-tagged Shiga toxins or toxoids with other proteins. Histidine tagging greatly facilitates purification of Shiga toxins, and the invention also relates to methods for purifying such toxins. The invention further relates to using the histidine-tagged Shiga toxoids or fusion proteins of Shiga toxoids as antigens for generating an immune response against infection or transmission by bacteria expressing Shiga toxin. It also relates to antibodies to Shiga toxins, toxoids, or Shiga toxin/toxoid fusion proteins, both monoclonal and polyclonal, and their use in treating, diagnosing, and preventing of disease and infections by pathogenic *E. coli*. Finally, the invention relates to preparing the Shiga toxins, toxoids, and fusion proteins.

BACKGROUND OF THE INVENTION

Enterohemorrhagic *Escherichia coli* (EHEC) are associated with food-borne outbreaks of bloody diarrhea or "hemorrhagic colitis" (HC) and the hemolytic uremic syndrome (HUS). (Spika, J. et al., "Hemolytic Uremic Syndrome and Diarrhea Associated with *Escheria coli*: 0157:H7 in a Day Care Center," *J. Pediatr.*, 109: 287-291(1986); Remis, R., "Sporadic case of hemorrhagic colitis associated with Escheria coli 0157:H7," *Ann. Intern. Med.*, 101:624-626 (1984); "Riley, L. et al., "Hemorrhagic

colitis associated with a rare *Escheria coli* serotype," *N. Engl. J. Med.*, 308:681-685 (1983)). EHEC infection can be deadly and poses a significant threat to the young and the elderly, who are the most likely to develop serious complications from EHEC infections. Several outbreaks and sporadic cases of HC and HUS have occurred over the past few years, with the largest outbreak in United States in 1993. In that outbreak, over 500 cases of HC and HUS were traced to contaminated hamburgers from a Jack-in-the Box fast food restaurant. (Centers for Disease Control and Prevention, *Morbid. Mortal. Weekly Rep.*, 42:258(1993)). In July 1996, a large outbreak of EHEC in Japan resulted in over 10,000 infected individuals and 8 deaths. Many Japanese children required hospitalization. Unfortunately, no cure or vaccine for HC and HUS is currently available.

Primarily, HC and HUS are transmitted by the ingestion of contaminated food, particularly undercooked beef products, such as hamburger. (Doyle et al., *J. Appl. Environ. Microbiol.* 53:2394 (1987); Samadpour et al., *J. Appl. Environ. Microbiol.* 60:1038 (1994)). With the prevalence of EHEC in cattle and the subjective nature of differentiating between cooked and undercooked hamburgers, a stop at a fast food restaurant or a family barbecue can result in tragedy. HC and HUS appear to be mediated by the toxin produced by EHEC and *Shigella dysenteriae* (for review see O'Brien and Holmes, *Microbiol.* Rev., 51: 206-220 (1987)). These bacteria produce a family of closely related cytotoxins that collectively will be called "Shiga toxins" for the purpose of this application. Shiga toxins (alternatively, "verotoxins") have cytotoxic, neurotoxic, and enterotoxic activity (Strockbine, N. et al., "Two toxin-converting phages from *Escheria coli* 0157:H7 strain 933 encode antigenically distinct toxins with similar biological activities," *Infect. Immun.*, 53:135-140 (1986)).

Based on their immunological cross-reactivity, the Shiga toxins have been divided into two groups. (Strockbine et al., *supra*). These groups have been designated Shiga toxin type 1 (Stx1) and Shiga toxin type 2 (Stx2). (Strockbine et al., *supra*; Calderwood et al., "Proposed New Nomenclature for SLT (VT) Family," ASM News, 62:118-119 (1996)). The Stx1 group contains the prototype Stx1 toxin from

EHEC as well as the Shiga toxin from *Shigella dysenteriae* type 1. In recent years, other types of toxins have been discovered and considered members of the Stx2 group. These are Stx2e, Stx2c, Stx2vha, and Stx2vhb. (Lindgren et al., *Infection and Immunology*, 61:3832 (1993); Schmitt, C. et al., "Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escheria coli* strains are responsible for the antigenic heterogeneity of the 0157:H strain E325II," *Infect. Immun.*, 59:1065-1073 (1991); Marques, L. et al., "*Escheria coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II," *FEMS Lett.*, 44:33-38 (1987)).

For the purposes of this application the term "Shiga toxin" encompasses Shiga toxin and any other toxins in the Stx1 or Stx2 group. The abbreviation "Stx" will refer to the protein designation, and the abbreviation *"stx"* to the gene designation.

These Shiga toxins do share similar genetic and protein organization, as set forth in Figure 1. The A subunit gene encodes the enzymatically active subunit. The A subunit polypeptide has two functional domains, A1 and A2, which are linked by a disulfide bond. The Al portion is an N-glycosidase that acts on the 28S rRNA subunit of eukaryotic ribosomes to inhibit protein synthesis. (Saxena, S. et al., "Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28S RNA when microinjected into *Xenopus* oocytes," *J. Biol. Chem.*, 264:596-601 (1989)). The A2 fragment is required for the binding of 5 B subunit polypeptides. The pentamer of B subunits is responsible for binding to a receptor on eukaryotic cells. A polypeptide containing the entire A subunit and B subunit pentameter is referred to as a Shiga holotoxin. Despite this knowledge about the toxin components, there is no known cure or vaccine for HC or HUS.

The need exists for therapeutic agents for the treatment and prevention of HC and HUS. However, progress in the search for such agents has been hampered by the lack of a fast and simple method for purifying Shiga toxins. Therefore, the need exists for such a fast and simple method. Moreover, the need exists for such a method that further allows for large-scale production of Shiga toxins while retaining their biological and immunological activity. The need also exists for such a method that

allows for large-scale production of Shiga toxoids and fusion proteins of Shiga toxins and toxoids. Such a method should simplify obtaining antibodies against Shiga toxin and vaccines against HC and HUS using Shiga toxoids and fusions of Shiga toxoids.

SUMMARY OF THE INVENTION

The present invention describes the isolation and purification of biologically and immunologically active histidine-tagged Shiga toxins (His-tagged), a toxin associated with HC and the potentially life-threatening disease HUS transmitted by strains of pathogenic bacteria. The present invention describes how his-tagging greatly simplifies and expedites purifying Shiga toxins, and describes an improved method for such purification.

One aspect of the invention is obtaining and using Shiga toxoids that are immunoreactive but not toxic. For example, the invention describes using such obtained Shiga toxoids in vaccines against HC and HUS.

Another aspect of the invention is obtaining and using fusion proteins of His-tagged Shiga toxins or toxoids. These fusion proteins have the advantage of combining beneficial properties of each protein, resulting, for example, in improved protein stability or targeted delivery of a his-tagged Shiga therapeutic agent.

Yet another aspect of the invention is obtaining and using antibodies to His-tagged Shiga toxins, toxoids, or Shiga toxin/toxoid fusion proteins. These antibodies can be either monoclonal or polyclonal and have potential uses in treating, diagnosing, or preventing HC and HUS caused by EHEC or *Shigella dysenteriae* type 1 infections.

Other aspects of the present invention will become apparent from the more detailed description provided below, to be read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the protein structure of Shiga toxin genes.

Figure 2 depicts the predicted amino acid sequence for the mature A subunit and the unprocessed B subunit of Stxl. (Calderwood et al., *Proc. Natl. Acad. Sci. USA*, 84: 4364-4368 (1987); DeGrandis et al., *J. Bacteriol.*, 169:4313-4319(1987)).

Figure 3 depicts the predicted amino acid sequence for the mature A subunit and the unprocessed B subunit of Stx2. (Jackson et al., *FEMS Lett.*, 44:109-114 (1987)).

Figure 4 depicts the predicted DNA sequence for *stx1* and DNA upstream of that sequence. (Calderwood et al., *Proc. Natl. Acad. Sci.* USA, 84: 4364-4368 (1987); DeGrandis et al., *J. Bacteriol.*, 169: 4313-4319 (1987)).

Figure 5 depicts the predicted DNA sequence for *stx2* and DNA upstream of that sequence. (Jackson et al., *FEMS Lett.*, 44: 109-114 (1987)).

Figure 6 depicts the approximately 1200 base pair fragments of *stx1* produced by PCR amplification. Figures 6a-c depict the fragments used to make plasmids pQHI, pQHEI, and p7HI, respectively. Nucleotides in lower case represent non-toxin sequences in the primers and/or base changes.

Figure 7 depicts the approximately 1200 base pair fragments of stx2 produced by PCR amplification. Figures 7a and 7b depict the fragments used to make plasmids pQHI and pQHEII, respectively. Nucleotides in lower case represent non-toxin sequences in the primers and/or base changes.

Figure 8 depicts the plasmid pQHI, encoding the His-Stx 1 fusion and driven by the T5 promoter.

Figure 9 depicts the plasmid pQHII, encoding the His-Stx 2 fusion and driven by the T5 promoter.

Figure 10 depicts the plasmid pQHEI, encoding the His-Enterokinase site-Stx1 fusion and driven by the T5 promoter.

Figure 11 depicts the plasmid pQHEII, encoding the His-Enterokinase site-Stx 2 fusion and driven by the T5 promoter.

Figure 12 depicts the plasmid pQHIIvhb, encoding the His-Stx 2 fusion and driven by the T5 promoter.

Figure 13 depicts the plasmid pQHEIIvhb, encoding the His-Enterokinase site-Stx 2 fusion and driven by the T5 promoter.

Figure 14 depicts the plasmid p7HI, encoding the His-Stx 1 fusion and driven by the PT7 promoter.

Figure 15 depicts the plasmid p7HII, encoding the His-Stx 2 fusion and driven by the PT7 promoter.

Figure 16 depicts the expression of His-Stx fusion proteins according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

An object of the invention is to purify large quantities of Shiga toxins that retain their biological and immunological properties. To achieve this object, a Shiga toxin gene was cloned into a Histidine-tag expression vector, expressed, and purified. An additional object of the invention is to obtain antigens specific to Shiga toxoids, a toxin that is non-toxinogenic but immunoreactive, for generating an immune response against Shiga toxins. Another object of the invention is the creation of antibodies against Shiga toxins or toxoids for treating, diagnosing, or preventing disease and infections by pathogenic bacteria. The his-tagged Shiga toxins or toxoids described above can be used for these purposes.

Those skilled in the art will also recognize that the size of the his-tagged Shiga toxin to be used may be varied according to the specific purpose for the Shiga toxin. For example, if the purpose is fusing the his-tagged Shiga toxin or toxoid with one or more proteins, a smaller fragment might be selected to enhance stability of the combined fusion product, although using a larger fragment is by no means precluded. The desired size of the His-Shiga toxin may also vary with the convenience of the available restriction sites, in light of the materials and methods known to those skilled in the art. Consequently, the terms "His-Shiga toxin" or "His-tagged Shiga Toxin" refers to the fragment of about 372-377 amino acids comprising the A and B subunits of any of the Shiga toxin family members fused with a histidine tag. Smaller fragments that retain biological and/or immunological function are also included. Biological function is measured by, for example, cytotoxicity to Vero cells, as described in Example III.A. Immunological function may also be tested by, for

example, neutralization by specific antisera, as described in Example III.B. A preferred embodiment of the invention is a His-Tagged Shiga holotoxin, containing 1 A subunit and 5 B subunits. In another preferred embodiment, the tag consists of six histidine residues. The most preferred embodiment is a His_6-Tagged Shiga holotoxin.

One of the objects of the present invention is to administer His-Shiga toxoids to protect against illness or disease caused by EHEC or *Shigella dysenteria* type I, such as HC and HUS. The object is achieved through the stimulation of immune response directed against Shiga toxins.

Consequently, the term "immunizing" or "immunization" is used in the application. The degree of protection achieved by such immunization will vary with the degree of homology between Shiga toxins and the His-Shiga toxoids, as well as other factors, such as unique attributes of the patient or the species treated. Moreover, immunization is not limited to avoiding infection altogether; it also includes decreasing the severity of the infection, as measured by the following indicators: reduced incidence of death, HUS, or permanent kidney damage; decreased levels of toxin; reduced fluid loss; or other indicators of illness regularly used by those skilled in the relevant art.

Unless specified otherwise, the uses and methods set forth herein are generally applicable to humans and animals. The term "patient" is used herein to mean both humans and animals, and "animals" is not limited to domesticated animals but also may include wildlife and laboratory animals.

Moreover, because His-Tagged Shiga toxins according to the invention have the biological and immunological properties of Shiga toxins, they may be used for any application appropriate for Shiga toxins. For example, it has been recently demonstrated that Stxl can be used to treat bone marrow cells from mice with human B-cell lymphomas. The Shiga toxin bound to the receptor on the lymphoma cell and the toxin killed the cancer cell. (LaCasse et al., *Blood* 88:1551(1996)). Thus, the skilled artisan would expect that His-Shiga toxins or fusions could be used for the same purpose and in the same manner.

Isolating and Purifying His-Tagged Shiga Toxin

The standard protocol for purification of Shiga toxin comprising A and B subunits uses biochemical techniques. The standard protocol was developed by O'Brien et al. (O'Brien et al., *Infect. Immun.* 40:675 (1983); O'Brien et al., *Infect. Immun.*, 30:170(1980)). The method employs four purification steps: 1) ammonium sulfate precipitation; 2) DEAE Sepharose column chromotography; 3) chromatofocusing; and 4) antibody affinity chromotography. This method has the advantages of employing publicly available materials, being capable of purifying all Shiga toxins, and being capable of purifying Shiga toxins for human use. Its disadvantage is that the minimum time required for this test is three weeks.

Another well-known method for purifying Shiga toxin from bacteria was developed by Keusch et al. (Donohue-Rolfe et al., *Infect. Immun.* 57:3888 (1989); Acheson et al., *Microb. Pathog.* 14:57 (1993)). This method employs a Shiga toxin receptor analog. The receptor analog is the P1 glycoprotein (P1gp) from tapeworm hydatid cysts material (HCM) in sheep gut. This method contains three purification steps: 1) ammonium sulfate precipitation; 2) Blue sepharose chromotography; and 3) Plgp column chromatography. The Plgp must be prepared from the HCM. Though faster than the standard method, this method still requires a minimum of two or more weeks. The hydatid cyst material must be obtained from infected sheep and is not publicly available. The method has the additional disadvantages of being capable of use with only those Shiga toxins that bind P1gp and, because of possible contamination, it is not appropriate for obtaining Shiga toxoids for use in humans.

Using recombinant methods, the Shiga toxin gene or portions of the Shiga toxin gene have been cloned and expressed in bacteria and purified. Zollman et al., *Prot. Expression Pur.* 5:291 (1994), purified a recombinant Stx1 A1 fragment. Acheson et al., *Infect. Immunol.* 63:301(1995), expressed and purified the Stx2 B subunit. Downes et al., *Infect. Immun.* 56:1929 (1988), expressed the *stx2* gene in bacteria and purified Stx2. However, the purification methods following expression

were essentially those of the standard method or hydatid cyst method and, therefore, had the same disadvantages.

In the search for a method for purifying Shiga toxins, applicants have developed a purification method based on the creation of a histidine-tagged Shiga toxin. Methods for histidine tagging are known in the art. For example, Fryxell et al., *Biochem. Biophys. Res. Comm.*, 210:253-259 (1995), added a kemptide and histidine tag to the A chain of the eukaryotic toxin ricin, which was later associated with the B subunit. The ricin toxin differs from Shiga toxins in origin (prokaryotic v. eukaryotic) and structure of the B subunit (the ricin B subunit is a single polypeptide, not a pentamer). Moreover, Fryxell et al. only expressed the A subunit with a His-Tag. In addition, Strauss et al., *FEMS Microbiol. Lett.*, 127:249-254 (1995), have his-tagged the C-terminus of the cholera toxin B subunit, and expressed a his-tagged B subunit-IgA protease fusion protein. However, this did not involve expressing the entire toxin with a His-Tag, and the expressed fusion protein did not undergo multimerization. Finally, Terbush & Novick, *J. Cell. Biol.*, 130:299-312 (1995), tagged the C-terminus of a multiunit yeast protein. This involves a eukaryotic rather than a prokaryotic system. Moreover, expressing a functionally active Shiga toxin requires retaining its multimer conformation, as well as its receptor binding and enzymatic activity.

Although His-Tagging of proteins is known, it was not expected that His-Tagging of a Shiga toxin would be successful. The skilled artisan would have believed that a His-Shiga toxin fusion would have lost cytotoxicity, because the skilled artisan would have expected that the attachment of a His-Tag to the amino acid terminus of a toxin would destroy its activity. Moreover, the multi-unit toxin would have been expected to be more susceptible to losing toxicity upon fusion with additional amino acids, since it is known that the toxin must retain its conformation for enzymatic activity and for binding of the B subunits to cell receptors, and the addition of amino acids would have been expected to destroy proper conformation. The skilled artisan would be aware that conformation and the charge of the molecule is critical to Shiga toxins. For example, the skilled artisan would know that deleting a few N-terminal

amino acids from Stx2A destroyed enzymatic activity, as reported in Perera et al., *Infect. Immunol.*, 59:829-835 (1991)). Similarly, altering the C-terminus of the B subunit affected toxicity. (Perera et al., *supra*). Additionally, Perera et al. suggested that the charge of the molecule plays an important role. The importance of preserving Shiga toxin conformation is further underscored by findings that the highly homologous Stx1A and Stx2B subunits cannot be combined to form an active toxin. (Weinstein et al., *Infect. Immun.*, 57:3743-3750 (1989)). Based on this knowledge, the skilled artisan would have expected that tagging a Shiga holotoxin with histidine residues would have unfavorably affected conformation and charge of the toxin product.

Surprisingly, his-tagging of Shiga toxin comprising A and B subunits generated a functional Shiga toxin, which has similar specific activity to Shiga toxin purified by standard methods. Moreover, the His-Shiga toxins are neutralized by monoclonal antibodies specific for Shiga toxins. Example I describes how to create the His-Shiga toxin fusion protein.

The following examples are intended to illustrate the invention but not to limit it. The skilled artisan will understand from these examples that modifications can be made that are still within the scope of the invention. The scope of the invention is defined by the claims.

Example I

A. Construction of Plasmid Encoding His-Tagged Shiga Toxin

The His-Stx fusion clones were generated by PCR amplification of *stx* operons, restriction enzyme digestion of the PCR products, and ligation of the fragments in-frame into the appropriate vectors. The expression vectors and primers were used to place histidine residues at the amino acid terminus of the toxins and place the constructs under the control of either an IPTG-inducible promoter (pQE vectors) (Qiagen, Inc., 9600 DeSoto Avenue, Chatsworth, CA 91311, 1-800-362-7737) or a T7 promoter (pt7-7) (Tabor et al., *Proc. Natl. Acad. Sci.* 82:1074 (1985)). The methods for obtaining His-Stx fusion clones are described in more detail below.

1. **Bacterial strains and plasmids**. The bacterial strains and plasmids used in this study are shown in Table 1.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristic(s)	Source or reference
E. coli strains		
DH5α	Host strain for cloning	BRL
XL1-Blue	Host strain for cloning; *lacI*; Tc^{r}	Stratagene
M15	Host strain for protein purification	Qiagen
Plasmids		
pJES120	Encodes *stx* II toxin operon	a
pJN25	Encodes *stx* I toxin operon	b
pSQ543	Encodes *stx* IIvhb operon	c
pQE30	Histidine fusion vector	Qiagen
pQE32	Histidine fusion vector	Qiagen
pREP4	*lacI*; Kn^{r}	Qiagen
pT7-7	T7 expression vector	d
pGP1-2	Encodes T7 RNA polymerase; KN^{r}	d

a = Lindgren et al., *Infect. Immunol.* 61:3832 (1993).
b= Newland et al., "Cloning of shiga-like toxin structural genes from a phage of *Escherichia coli* strain 933, in *Advances in Research on Cholera and Diarheas* (S. Kuwahara & N.F. Pierce eds. 1994).
c= Lindgren et al., *Infect. Immunol.* 62:623 (1994).
d= Tabor et al., *Proc. Natl. Acad. Sci. USA* 82:1074 (1985).

2. **Media and enzymes.** Bacterial strains were grown in L broth (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl). Kanamycin, tetracycline, and ampicillin (Sigma Chemical Co., St. Louis, MO.) were added to the medium at final concentrations of 25, 10, and 100 μg/ml (respectively) as needed. Restriction

endonucleases, calf intestinal phosphatase, and ligase were from Boehringer Mannheim, Indianapolis, Ind., or U.S. Biochemicals Corporation, Cleveland, Ohio. Enzymes were used according to manufacturer's instructions.

3. **Primers and PCR.** The Shiga toxin genes are cloned using polymerase chain reaction (PCR), a standard technique in the art. Primers were designed to amplify the *stx* toxin operons beginning at the first codon of the mature A subunit gene and ending downstream of the termination codon of the B subunit gene and created using standard techniques. The primers contained recognition sequences to generate unique restriction sites at the ends of the toxin operon. In a preferred embodiment, the 5' primers also contained sequences to encode the recognition sequence of the protease enterokinase to allow for removal of the histidine residues. The primers used are shown in Table 2.

TABLE 2. Primers used.

Primer	Primer Sequent (5'-3')	Restriction site
HEC	GCGGATCCGATGACGATGACAAACGGGAGTTTACGATAGACTT	BamHI
HBAM	GCGGATCCGGGAGTTTACGATAGACTT	BamHI
HH3	CCACGAATAAGCTTATGCCTCA	HindIII
IBAM5	GCGGATCCAAGGAATTTACCTTAGACTTC	BamHI
IEC5	GCGGATCCGATGACGATGACAAAAAGGAATTTACCTTAGACTTC	BamHI
IPST3	ATTTTCACTGCAGCTATTCTG	PstI
SLTIIH5	GCATATGCATCACCATCACCATCACCGGGAGTTTACGATAGAC	NdeI
SLTIH5	GCATATGCATCACCATCACCATCACAAGGAATTTACCTTAGECTTC	NdeI
SLTLIC3	TAACATTTATCGATATCTCCGCCTG	ClaI

Sequences encoding *stx* toxins were amplified from toxin clones using a PCR kit (GeneAmp kit, Perkin-Elmer Cetus, Norwalk, CT), which was used according to the manufacturer's instructions. The resulting *stx* PCR products were approximately

1200 bp are shown in Figures 6a, 6b, 6c, 7a, and 7b. The DNA products contain the coding sequences for the mature A subunit and the unprocessed B subunit.

Procedures for cloning are well known in the art and are described in Maniatis, *Molecular Cloning: A Laboratory Manual* (1982)).

4. **DNA manipulations.** Plasmid DNA was isolated by the method of Holmes and Quigley, *Anal. Biochem.*, 114:193-197 (1981). Alternatively, plasmid DNA was purified using Qiagen columns (Qiagen Inc., Chatsworth, CA). PCR products were digested with restriction endonucleases and ligated into the pQE30/32 vectors (Qiagen, Inc.), or into the vector pT7-7. PCR reactions and ligations are summarized in Table 3.

TABLE 3: PCR Reactions and ligations.

Plasmid template	Primer pair	Vector	Cloning sites	Resulting clone
pJES120	IIEC + IIH3	pQE30	BamHI/HindIII	pQHEII
pJES120	IIBAM + IIH3	pQE32	BamHI/HindIII	pQHII
pJN25	IEC5 + IPST3	pQE30	BamHI/PstI	pQHEI
pJN25	IBAM + IPST3	pQE30	BamHI/PstI	pQHI
pJES120	SLTIIH5 + IIH3	pT7-7	NdeI/HindIII	p7HII[a]
pJN25	SLTIH5 + SLTIC3	pT7-7	NdeI/ClaI	p7HI
pSQ543	IIEC + IIH3	pQE30	BamHI/HindIII	pQHEIIvhb[a]
pSQ543	IIBAM + IIH3	pQE32	BamHI/HindIII	pQHIIvhb[a]

[a] The construction of these plasmids is in progress.

To illustrate, the clone pQHEII was constructed as follows:

Plasmid pJESl20 was the template with primers IIEC and IIH3 in a PCR reaction for amplification of the *stx2* operon. The resulting PCR product started with the first codon of the mature A subunit gene, extended through the A subunit gene, the complete B subunit gene, and ended just downstream of the terminate codon of the B subunit gene (Figure 6b). The PCR product was digested with the restriction endonucleases Bam HI and Hind III, as was the vector plasmid pQE30. The vector

pQE30 was chosen because ligation of the PCR product with pQE30 at the BamHI sites would result in an in-frame protein fusion of the 6 histidine residues, the enterokinase cleavage site, additional amino acids, and the +1 residue of the mature A subunit. The digested PCR product was ligated into the digested vector pQE30. The ligation reaction was transformed into strain XL1-Blue and plated on agar that contained ampicillin. Colonies were screened for the presence of a plasmid that contained an approximately 1200 bp BamHI/HindIII DNA insert. Clones were confirmed by IPTG induction of toxin expression (Example II) with a subsequent test for cytoxicity on vero cells (Example III). Positive clones were then transformed into M15(pREP4) for large scale production of toxin.

Example II

Large scale purification of His-Tagged Shiga Toxins

His-Shiga toxin was purified under nondenaturing conditions because of the multi-subunit nature of the Shiga toxins. The strain was streaked onto a selective agar plate and incubated at 37°C for 18-24 hrs. A 20 ml overnight culture was then prepared from a colony. The saturated culture was then diluted 1/50 into one liter of L broth with antibiotics and the culture was grown at 37°C until it reached an $O.D._{600}$ of 0.7-0.9. IPTG (2mM final concentration) was then added to induce expression of the His_6 tagged toxin and the culture was grown for an additional 5 hrs. Cells were pelleted and the pellet was kept at -70°C overnight. The pellet was resuspended in sonication buffer (50 mM sodium phosphate (pH 8.0), 300 mM sodium chloride, 20 mM imidazole, 30 μg/ml PMSF), and the cells were sonicated to release toxin. Alternatively, the cells were treated with polymixin-B (2 mg/ml final concentration) for 3 hrs at 4°C. The extracts were clarified by centrifugation and filtered through a milipore 0.45 μm filter.

The nickel-nitrilotriacetic acid ligand (Ni-NTA) gel was equilibrated with sonication buffer and the cell extract was added to the gel. Protein was allowed to bind for 1 hr at room temperature or at 4°C. The gel was washed with sonication buffer followed by wash buffer (50 mM sodium phosphate (pH 8.0), 300 mM sodium

chloride, 20 mM imidazole, 30 µg/ml PMSF, 10% glycerol, 1% tween-20). Protein was eluted from the gel with a gradient of imidazole (0-500 mM in wash buffer without tween-20), and 1 ml fractions were collected.

Fractions were tested for cytotoxicity on Vero cells (as explained in Example III) and were subjected to SDS-PAGE and silver stain. Fractions that were highly cytotoxic and relatively clean were pooled and dialyzed against sonication buffer. This pool was then placed onto a Ni-NTA spin column (Qiagen) to further purify the His_6-toxin and the resulting two fractions were dialyzed against PBS. A final cytotoxicity assay and BCA protein assay were performed for the determination of the specific activity of the purified toxin.

The protocol described above is modification of the non-denaturing protocol described by Qiagen to purify His-tagged proteins. However, the toxin that eluted contained many contaminants. To achieve purer His-Shiga toxin, modifications were made. Specifically, Tween-20 was added to the wash buffer, and the pH of the wash buffer was adjusted to 8. Also, a final Ni-NTA spin column was added.

This one-step His-affinity method for purifying His-Shiga toxin by an Ni-NTA column has several advantages over existing methods, as summarized in Table 4.

TABLE 4. Comparison of Toxin Purification Techniques

Purification Method	Minimum time required[a]	Steps[b]	Materials available	Use for all Shiga toxins
Standard	3 weeks	4	yes	yes
Hydatid Cyst	2 weeks +	3	no	no
His_6 affinity	1 week	2	yes	yes

[a] Time from streaking the strain onto an agar plate. This does not include the preparation of Plgp from hydatid cyst material which takes a minimum of 1.5 weeks.
[b] This does not include the multiple steps involved in the purification of Plgp from hydatid cyst material and preparation of the column.

The Ni-NTA one-step method is superior because of its relative speed and simplicity. It requires a minimum of one week as opposed to a minimum of two or

more weeks. Moreover, all of the materials are readily available, the method is not limited to Shiga toxins that bind P1gp, and the products are suitable for use in humans.

The Shiga toxin obtained by the method has many uses. For example, the His-Shiga toxin may be used as a positive control antigen in a Shiga toxin detection kit. Such kits will use a purified His-Shiga toxin as positive indicator for the toxin in a sample. Other uses are detailed in the Examples below.

Example III

Verifying Biological and Immunological Activity of His-Shiga Toxins

A. Vero Cell Cytotoxicity Assay

The cytotoxicity of His-Shiga toxins obtained according to the methods described in Examples I and II was verified by determining their cytotoxicity for Vero cells. Cytotoxicity assays on strains that expressed His-Shiga toxins were done essentially as described by Gentry and Dalrymple, *J. Clin. Microbiol*, 12: 361-366 (1980). Briefly, cultures induced for the expression of His-Shiga toxins were disrupted by sonic lysis and clarified by centrifugation. The extracts were serially diluted in tissue culture medium (Dulbecco modified Eagle medium containing 10% fetal calf serum, 0.8 mM glutamine, 500 U of penicillin G per ml, and 500 mg of streptomycin per ml). One hundred microliters of 10-fold dilutions of the lysates were added to microtiter plate wells containing about 10^4 Vero cells in 100 μl of medium. The tissue culture cells were incubated at 37°C in 5% CO_2 for 48 hours and then fixed and stained with crystal violet. The intensity of color of the fixed and stained cells was measured with a Titertek reader at 620 nm.

B. Antisera Neutralization Assay

His-Shiga toxins obtained according to the methods described in Examples I and II were tested for antisera neutralization. Neutralization of cytoxic activity was described in great detail in Schmitt et al., *Infect. and Immun.*, 59:1065-1073 (1991). Briefly, lysates were incubated with serial dilutions of monoclonal or polyclonal antisera specific for Stxl or Stx2 at 37°C for 2 hours. One hundred microliters of the

samples were then added to vero cells as described above. Percent neutralization was determined by the following formula:

$$\{[A_{620}(\text{toxin + antibody}) - A_{620}(\text{toxin})]/A_{620}(\text{untreated cells})\} \times 100.$$

Example IV

Constructing Fusions with His-Shiga Toxins and Other Proteins

Using methods well-known in the art, the His-Shiga toxin could be fused with another protein of interest. These methods include chemical and genetic methods, as in cloning and expressing a fusion protein, although one skilled other methods are readily apparent to one skilled in the art. (D.V. Goeddell, *Meth. Enzymol.* Vol. 185(1990); Itakura, *Science* 198:1056 (1977)). For example, if a combination vaccine for immunization against Shiga toxin and another toxin (protein X) is desired, then these two toxins can be fused into a single protein. This can be achieved by first cloning the codons for the histidine residues in frame to the coding region of protein X. The fragment containing His-Protein X is then subcloned in-frame of the Shiga toxin operon. In a preferred embodiment, the fragment is subcloned in-frame to the A2-B portions of the Shiga toxin operon. The resulting His-Protein X-A2-B5 fusion would ideally result in immunization against Shiga toxin and protein X.

One skilled in the art would recognize that various proteins from pathogens and haptens may be conjugated to a His-Shiga toxin. Haptens and antigens may derive from but are not limited to bacteria, rickettsiae, fungi, viruses, parasites, drugs, or chemicals. They may include, for example, small molecules such as peptides, oligosaccarides, and toxins. Certain antimicrobial drugs, chemotherapeutic drugs having the capacity of being absorbed into the intestine may also be coupled to Shiga toxin for targeted delivery, since the B subunit pentamer binds to receptors in the intestine. Conjugation methods are well known in the art. Exemplary methods are set forth in Goeddel, "Systems for Heterologous Gene Expression," *Meth. Ezymol.*, 185 (1990), Itakura, "Expression in *E. coli* of a chemically synthesized gene for the hormone somatostatin," *Science*, 198:1056-1063 (1977), and Goeddel et al.,

"Expression of chemically synthesized genes for human insulin," *Proc. Natl. Acad. Sci.* USA, 281: 544-548(1979).

Conjugation may be achieved by genetically fusing His-Shiga toxoids by standard molecular techniques or by conjugation to a polysaccharide. Methods of conjugation include those outlined in M. Brunswick et al., *J. Immunol*, 140:3364 (1988) and Chemistry of Protein Conjugates and Crosslinking, *CRC Press*, Boston (1991). Coupling of Shiga toxids to other proteins or polysaccharides would prevent disease from additional pathogens.

Example V

His-Shiga Toxoids

A form of Shiga toxin that is immunoreactive but not toxinogenic is needed for immunization in animals. Such a His-Shiga toxoid can be generated using chemical or genetic methods. The chemical method involves treating the His-Shiga toxin with either formaldehyde or glutaraldehyde, as described by Perera et al., *J Clin. Microbiol.* 26:2127 (1988)). Briefly, samples of toxin containing 100 μg of protein are treated for 3 days at 37°C with 0.1 M Na_2HPO_4 (pH 8.0) containing 1% formaldehyde, and the residual formaldehyde is removed by dialysis against phosphate-buffered saline (PBS). To prepare His-Shiga toxoid by treatment with glutaraldehyde, crude toxin samples containing 50 μg of protein are incubated at 37°C in 0.11% glutaraldehyde in 0.1 M Na_2HP0_4 (pH 8.0) for 30 min. The toxoid is then tested on Vero cells, as described in Example III, for loss of cytotoxicity.

Genetically, a toxoid may be produced by site-directed mutagenesis, as described in Gordon et al., *Infect. Immun.* 60:485 (1992); Hovde et al., *Proc. Natl. Acad. Sci.* 85:2568 (1988); Jackson et al., *J. Bacteriol.* 172: 3346-3350 (1990). Several methods and kits exist for site-directed mutagenesis of a gene. One method employs the Bio-Rad Muta-Gene *in vitro* mutagenesis kit. Oligonucleotides can be designed and synthesized which alter specific condons in the toxin genes. Uracil-incorporated, single-stranded target plasmid DNA will be mutagenized according to

the directions supplied by the manufacturer of the mutagenesis kit. The nucleotide changes are then confirmed by DNA sequence analysis.

In the His-shiga toxin, two or more amino acids essential for enzymatic activity should be altered. For example, A subunit targets are the residues E167 and E170. The Shiga toxoid resulting from this mutation has been used for vaccinating pigs. (Gordon et al., *supra*).

Example VI

Passive Immunity to Shiga Toxin Using His-Shiga Toxin and Toxoid

A. Antisera Specific for His-Shiga Toxins

Antisera specific for Shiga toxins are required to treat and prevent potentially-deadly infections by EHEC and *Shigella dysenteriae* type I. Specifically, once a child becomes infected, the child and his or her family members or children in his or her day care group can receive anti His-Shiga toxin sera to achieve a protective immune response. A protective immune response is one that elicits sufficient antibody to permit a patient to avoid infection, decrease the significance or severity of an infection, or decrease the ability of bacteria to colonize the gastrointestinal tract.

Animal studies have shown that administering anti-Shiga toxin sera to mice results in resistance to normally lethal infection of EHEC. (Lindgren et al., *Infect. Immun.* 62:623(1994); Wadolowski et al., *Infect. Immun.* 58:3959). Thus, applicants believe that administering anti-Shiga toxin sera to humans and other mammals would result in a protective immune response against Shiga toxin infections.

Methods are well-known in the art for producing antisera for passive immunization. For example, His-Shiga toxoid, obtained by the methods described in Example VI, can be administered to a mammal, such as a horse intraperitoneally. Currently, the horse is used to produce serum against botulism toxin for administration to humans, Hibbs et al., *Clin. Infect Dis.*, 23:337-40 (1996), and the horse would be a preferred method for producing shiga toxin antiserum. After several boosts with His-Shiga toxoid, the serum of the immunized horse (or other mammal) would be tested for neutralizing the cytotoxicity of Shiga toxins. Advantageously, a large amount of

serum can be quickly made using this method. However, patients must first be screened for an immune reaction to horse serum. For this purpose, a small amount of horse serum would be subcutaneously injected, and the patient would be monitored for a reaction. Such methods for administering horse antiserum against toxins to humans are well known to the skilled artisan. Hibbs et al., *supra*; Dehesa and Possani, *Toxicon*, 32: 1015-1018(1994); Gilan et al., *Toxicon*, 27:1105-1112 (1989).

More preferably, the His-Shiga toxoid can be administered to human volunteers, either intraperitoneally or orally. The plasma from these volunteers is then isolated, and the human anti-His-Shiga toxin serum can be administered to patients. No threat of serum sickness arises from this method. Human hyperimmune globulin to Hemophilus influenzae b, Streptococcus pneumoniae, and Neisseria meningitidis has previously been prepared by others (Siber et al., *Infect. and Immun.*, 45: 248-254 (1984)).

B. Vaccines Against Shiga Toxins

An embodiment of the invention is vaccines against Shiga toxin infection. For example, these vaccines can include antibodies directed against His-Shiga toxin, obtained further described in Example VII. Moreover, these vaccines can be combination vaccines that comprise His-Shiga toxoid fused or conjugated with another protein, hapten, or antigen, as described in Example IV. These vaccines can be administered intraperitoneally or injectably by methods well known in the art.

A preferred method of administering His-Shiga toxin or toxoid and fusions thereof is by further conjugation to Synsorb® (SynSorb Biotech, Inc., 1204 Kensington Road, N.W., Calgary, Alberta, Canada, T2N3P5.) Synosorb is a sand-like material to which Shiga toxin receptor (Gb3) is covalently bound (Armstrong et al., *J. Infect. Dis.*, 171:1042 (1995)). This compound has been shown to bind Shiga toxins and appears to be safe for human ingestion. (Armstrong et al., *supra*) The Synsorb is bound to the B subunit pentamer via the B subunit pentamer-receptor reaction. Conjugation with Synsorb adds further stability.

Another embodiment of the invention involves the administration of nucleic acid vaccines. DNA encoding a His-Shiga toxoid is injected into a patient as naked DNA, or the DNA is delivered to the body by a carrier system such as retro viruses, adenoviruses, or other carriers known in the art. Following administration, the patient mounts an immune response against transiently expressed foreign antigens.

Currently nucleic acid vaccines, in general, are all nearing clinical trials. This approach to vaccines involves delivering the DNA encoding the desired antigen into the host by inserting the gene into a nonreplicating plasmid vector (Marwick, C. JAMA 273:1403(1995); reviewed in Vogel, F.R. and N. Sarver, *Clin. Microbiol.*, Rev. 8:406 (1995)).

The first published demonstration of the protective efficacy of such a vaccine has shown that intramuscular injection of plasmid DNA encoding influenza A virus (A/PR/8/34) nucleoprotein (NP) elicited protective immune responses in BALB/c mice against a heterologous strain of influenza virus (A/HK/68) (Ulmer, J.B. et al. *Science* 259:1745(1993)). Immunized animals had reduced virus titers in their lungs, decreased weight loss, and increased survival compared with challenged control mice. Both NP-specific cytotoxic T lymphocytes (CTL's) and NP antibodies were generated. The NP antibodies were ineffective at conferring protection, but the CTL's killed virus-infected cells and cells pulsed with the appropriate major histocompatibility complex class I-restricted peptide epitope.

Another study has shown that intramuscular injection of plasmid DNA encoding influenza virus A/PR/8/34 hemagglutinin resulted in the generation of neutralizing antibodies that protected mice against a heterologous lethal influenza virus challenge (Montgomery, D.L. et al. *DNA Cell Biol.*, 12:777 (1993)).

Example VII

His-Shiga Antibodies

His-Shiga antibodies, polyclonal and monoclonal, can also be used in the treatment, diagnosis, and prevention of infections related to Shiga toxins. Because of their increased specificity, monoclonal antibodies are preferred. His-Shiga toxin

antibodies can be administered to humans or other mammals to achieve a protective immune response, for treatment or prophylaxis. Antibodies, in a physiologically acceptable carrier, may be administered orally or intraperitoneally. For this purpose, monoclonal antibodies are preferred and humanized monoclonal antibodies are particularly preferred. Positive clinical responses in humans have been obtained with monoclonal antibodies, and one skilled in the art would know how to employ Shiga monoclonal antibodies in humans. *See* Fagerberg et al., "Tumor Regression in Monoclonal Antibody-treated Patients Correlates with the Presence of Anti-idiotype-reactive T Lymphocytes," *Cancer Research*, 55:1824-27 (1995); "A Phase I Study of Human/Mouse Chimeric Anti-ganglioside GD2 Antibody ch14.18 in Patients with Neuroblastoma," *Eur. J. Cancer*, 2:261-267 (1995)).

Another embodiment of the invention involves using antibodies to diagnose Shiga toxin infections. The antibody, using well-known methods of immunoassaying, is brought into contact with a sample from a patient, such as a fecal sample. In addition, the antibody may be used to detect Shiga toxins in sample taken from cow, such as cow feces. Moreover, meat may be tested using the anti His-Shiga toxin antibody for detection. A detection kit comprising the His-Shiga toxin antibody can be used for this purpose.

For example, a sandwich Elisa can be used. In this kit, rabbit anti-His-Shiga toxin antibody can be used to capture toxin from a sample to be tested. Goat anti-His-Shiga toxin antibody can then be added followed by a secondary antibody such as mouse α-goat antibody conjugated to horseradish peroxidase. The antibody can be detected by standard methods.

His-Shiga toxin polyclonal antibodies and monoclonal antibodies are described below.

A. Making Polyclonal Antibodies

The technique of Harlow, E. and D. Lane (eds.), *Antibodies- a Laboratory Manual*, Cold Spring Harbor, New York (1988), may be followed. The general procedure is outlined herein. Take pre-bleeds of each mouse to be immunized: Bleed

from the tail vein into an eppendorf tube. Incubate at 37°C for 30 min, stir gently with a sterile toothpick (to loosen the clot), store overnight at 4°C. In the morning, spin 10 min/10,000 rpm in the microfuge, and collect the serum (i.e., supernatant; red blood cells are the pellet). Store the serum at -20°C. The sera obtained will be used as a negative control after the mice are immunized.

Inject a BALB/c mouse intraperitoneally with 25 μg of His-Shiga toxoid (using Titremax adjuvant, according to the instructions of the manufacturer (CytRyx Corp., 154 Technology Pkwy., Norcross, GA. 30092, 800-345-2987)). Wait 2 weeks, boost with an identical shot, wait 7 days and bleed from the tail vein into an eppendorf tube. Incubate at 37°C for 30 min, stir gently with a sterile toothpick (to loosen the clot), store overnight at 4°C. In the morning, spin 10 min/10,000 rpm in the microfuge, and collect the serum. Store the sera at -20°C.

B. ELISA to test titer of Abs.

The technique of Harlow, E. and D. Lane (eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor, New York (1988), may be followed. The general procedure is outlined below:

(1) bind His-Shiga toxoid to plastic microtiter plates at 50 ng/well in PBS. Incubate 2h/RT (room temp) or overnight at 4°C.

(2) wash plate 2X with PBS.

(3) block wells with 100 μl blocking solution [3% bovine serum albumin (Sigma Chemical, St. Louis, MO.), 0.02% sodium azide (Sigma) in PBS - store stock at 4°C] for 1 - 2 h at RT.

(4) wash plate 2X with PBS.

(5) primary Ab = 50 μl test sera diluted in blocking solution for example, start with 1:50 and do eleven 1:2 dilutions, or start with 1:50 and do eleven 1:10 dilutions), incubate 2 h/RT.

(6) wash 4X with PBS.

(7) secondary Ab = goat horseradish-conjugated anti-mouse Ig, affinity purified (Boehringer Mannheim Corp., 9115 Hague Rd., P.O. Box 50414, Indianapolis, IN.

46250,800-262-1640). Add secondary Ab diluted 1:500 in blocking solution without azide. Incubate 1 h/RT.

(8) wash 4X with PBS.

(9) add 100 μl TMB Peroxidase substrate to each well (prepared according to the instructions of the manufacturer, BioRad Labs, 3300 Regatta Blvd., Richmond, CA. 94804). Allow blue color to develop (no more than 10 min). Stop the reaction with 100 μl H_2SO_4. Read the plate at 450 nm.

A titer is defined as an absorbance value ≥0.2 units above that obtained for mouse preimmune sera.

Anti-Shiga toxin Abs obtained from animals may be used clinically if one changes the specificity of the antibody to human. Such techniques are well known to those of ordinary skill in the art. G. Winter et al., "Man-made antibodies," *Nature*, 349: 293-299 (1991); P.T. Jones et al., "Replacing the complementarity-determining regions in a human antibody with those from a mouse," *Nature*, 321: 522-525 (1986); P. Carter et al., "Humanization of an anti-p185^{HER2} antibody for human cancer therapy," *Proc. Natl. Acad Sci. USA*, 89: 4285-4289 (1992). Such antibodies may be given to the sibling of an infected patient to reduce the risk of infection of the sibling.

C. Raising Monoclonal Antibodies to His-Shiga Toxin

Monoclonal antibodies directed against Shiga toxin are used to passively protect a patient against EHEC and *Shigella dysenteriae* type I infections. Monoclonal antibodies are generated from mouse cells, and the specificity of these antibodies are changed for use in humans. G. Winter et al., "Man-made antibodies," *Nature*, 349: 293-299 (1991); P.T. Jones et al., "Replacing the complementarity-determining regions in a human antibody with those from a mouse," *Nature*, 321: 522-525 (1986); P. Carter et al., "Humanization of an anti-pl85^{HER2} antibody for human cancer therapy," *Proc. Natl. Acad Sci. USA*, 89: 4285-4289 (1992). Monoclonal Abs represent a more "pure" antibody for administration to a patient.

The procedure outlined in Harlow, E. and D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor, New York (1988) is followed: Five 4- to 5-week old

female BALB/cJ mice are prebled, and immunized intraperitoneally with 25 μg His-Shiga toxoid suspended in 100 μl of TiterMax. Mice are boosted twice in two week intervals, intraperitoneally with 25 μg His-Shiga toxoid suspended in 100 μl of TiterMax. Seven days after each boost, blood (~300 - 500 μl) is collected from the tail vein. Sera are assayed for the presence of anti-Shiga toxin antibody by ELISA (as described above).

Mice producing high titers of anti-His Shiga toxin antibodies are boosted both intravenously and intraperitoneally with 25 μg of His-Shiga toxoid in 100 μl of PBS, sacrificed three days later, and sera collected. Spleen cells are isolated and fused to Sp2/0-Ag mouse myeloma cells (ATCC #CRL1581) at a ratio of 10 spleen cells to 1 myeloma cell. Fused cells are distributed into microdilution plates, and culture supernatants are assayed by ELISA after 3-4 weeks of culture for anti-His-Shiga toxin antibodies. Cultures positive for production of anti-His Shiga toxin antibodies are expanded and cloned twice by limiting dilution.

The person skilled in the art would understand how to use and practice the invention based on the above disclosure. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

We claim:

1. A polypeptide comprising a Shiga toxin having a histidine tag.

2. A polypeptide comprising an immunoreactive but non-toxinogenic form of the polypeptide of claim 1.

3. A fusion protein comprising the polypeptide of claim 1 or 2 fused to a second polypeptide or a portion thereof.

4. A method for large-scale isolation and purification of Shiga toxin comprising the steps of:

a) expressing Shiga toxin with a histidine tag in bacteria; and

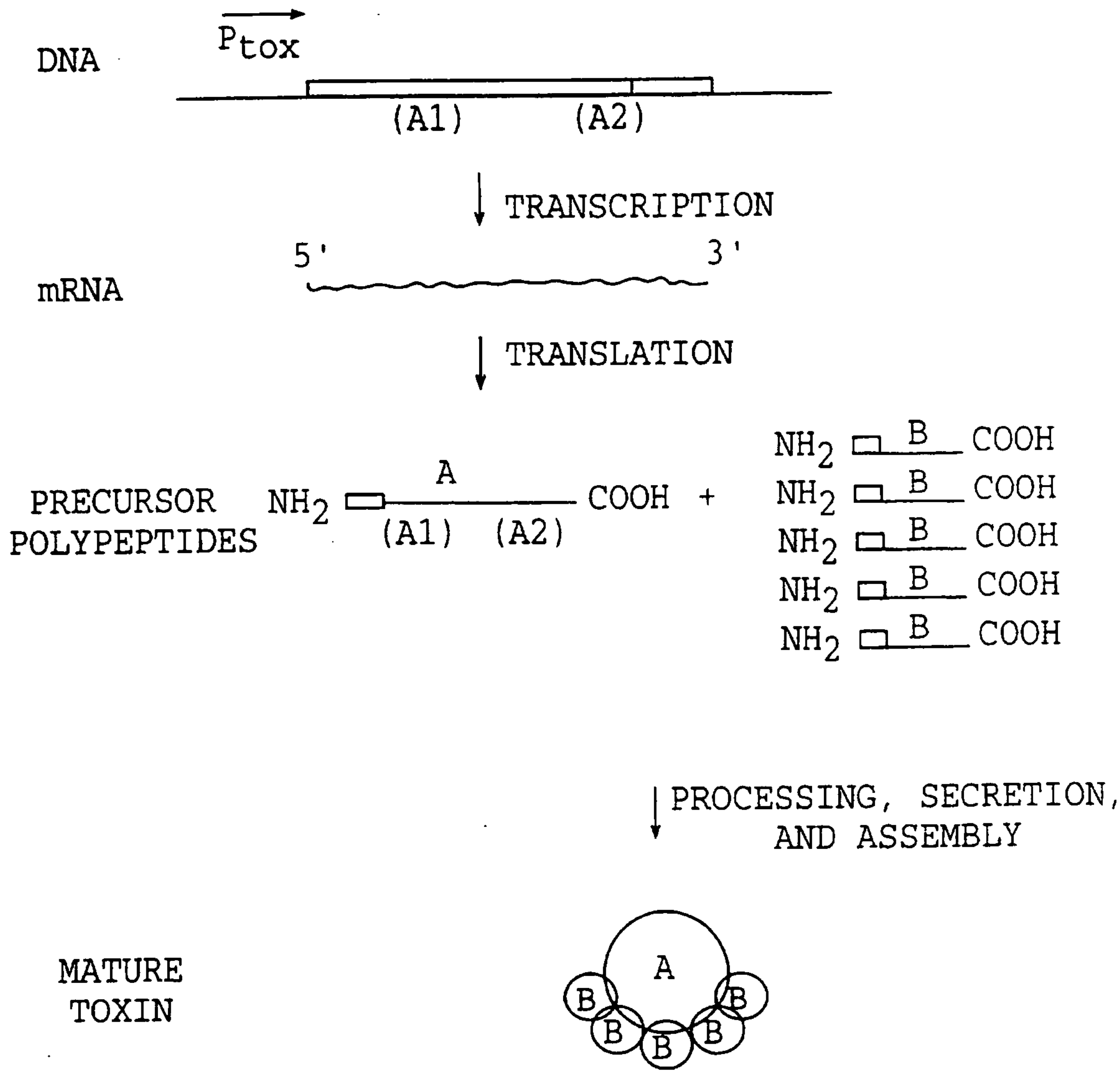
b) eluting cell extract containing histidine-tagged Shiga toxin over a nickel-nitrilotriacetic acid ligand (Ni-NTA) gel.

5. A method of providing passive immune protection comprising the step of administering antisera directed against the polypeptide of claim 2 to patients in need thereof.

6. A method of treating infections mediated by toxins of the Shiga toxin family comprising the step of administering antibodies against the polypeptide of claim 2 to patients in need thereof.

7. A vaccine comprising an antibody directed against the polypeptide of claim 2.

8. A vaccine comprising a nucleotide encoding the polypeptide of claim 2.



$\overrightarrow{P}$, PROMOTER. ☐, SIGNAL SEQUENCE.

FIG. 1

Stx 1A

1	KEFTLDFSTA	KTYVDSLNVI	RSAIGTPLQT	ISSGGTSLLM	IDSGSGDNLF
51	AVDVRGIDPE	EGRFNNLRLI	VERNNLYVTG	FVNRTNNVFY	RFADFSHVTF
101	PGTTAVTLSG	DSSYTTLQRV	AGISRTGMQI	NRHSLTTSYL	DLMSHSGTSL
151	TQSVARAMLR	FVTVTAEALR	FRQIQRGFRT	TLDDLSGRSY	VMTAEDVDLT
201	LNWGRLSSVL	PDYHGQDSVR	VGRISFGSIN	AILGSVALIL	NCHHHASRVA
251	RMASDEFPSM	CPADGRVRGI	THNKILWDSS	TLGAILMRRT	ISS*

Stx 1B

1	MKKTLLIAAS	LSFFSASALA	TPDCVTGKVE	YTKYNDDDTF	TVKVGDKELF
51	TNRWNLQSLL	LSAQITGMTV	TIKTNACHNG	GGFSEVIFR*	

FIG. 2

Stx 2A

1	REFTIDFSTQ	QSYVSSLNSI	RTEISTPLEH	ISQGTTSVSV	INHTPPGSYF
51	AVDIRGLDVY	QARFDHLRLI	IEQNNLYVAG	FVNTATNTFY	RFSDFTHISV
101	PGVTTVSMTT	DSSYTTLQRV	AALERSGMQI	SRHSLVSSYL	ALMEFSGNTM
151	TRDASRAVLR	FVTVTAEALR	FRQIQREFRQ	ALSETAPVYT	MTPGDVDLTL
201	NWGRISNVLP	EYRGEDGVRV	GRISFNNISA	ILGTVAVILN	CHHQGARSVR
251	AVNEESQPEC	QITGDRPVIK	INNTLWESNT	AAAFLNRKSQ	FLYTTG

Stx 2B

1	MKKMFMAVLF	ALASVNAMAA	DCAKGKIEFS	KYNEDDTFTV	KVDGKEYWTS
51	RWNLQPLLQS	AQLTGMTVTI	KSSTCESGSG	FAEVQFNND*	

FIG. 3

1 CTTGACCAGATATGTTAAGGTTGCAGCTCTCTTTGAATATGATTATCATTTTCATTACGT 60

61 TATTGTTACGTTTATCCGGTGCGCCGTAAAACGCCGTCCTTCAGGGCGTGGAGGATGTCA 120

121 AGAATATAGTTATCGTATGGTGCTCAAGGAGTATTGTGTAATATGAAAATAATTATTTTT 180

181 AGAGTGCTAACTTTTTTCTTTGTTATCTTTTCAGTTAATGTGGTGGCGAAGGAATTTACC 240

241 TTAGACTTCTCGACTGCAAAGACGTATGTAGATTCGCTGAATGTCATTCGCTCTGCAATA 300

301 GGTACTCCATTACAGACTATTTCATCAGGAGGTACGTCTTTACTGATGATTGATAGTGGC 360

361 TCAGGGGATAATTTGTTTGCAGTTGATGTCAGAGGGATAGATCCAGAGGAAGGGCGGTTT 420

421 AATAATCTACGGCTTATTGTTGAACGAAATAATTTATATGTGACAGGATTTGTTAACAGG 480

481 ACAAATAATGTTTTTTATCGCTTTGCTGATTTTTCACATGTTACCTTTCCAGGTACAACA 540

541 GCGGTTACATTGTCTGGTGACAGTAGCTATACCACGTTACAGCGTGTTGCAGGGATCAGT 600

601 CGTACGGGGATGCAGATAAATCGCCATTCGTTGACTACTTCTTATCTGGATTTAATGTCG 660

661 CATAGTGGAACCTCACTGACGCAGTCTGTGGCAAGAGCGATGTTACGGTTTGTTACTGTG 720

721 ACAGCTGAAGCTTTACGTTTTCGGCAAATACAGAGGGGATTTCGTACAACACTGGATGAT 780

781 CTCAGTGGGCGTTCTTATGTAATGACTGCTGAAGATGTTGATCTTACATTGAACTGGGGA 840

841 AGGTTGAGTAGCGTCCTGCCTGACTATCATGGACAAGACTCTGTTCGTGTAGGAAGAATT 900

901 TCTTTTGGAAGCATTAATGCAATTCTGGGAAGCGTGGCATTAATACTGAATTGTCATCAT 960

961 CATGCATCGCGAGTTGCCAGAATGGCATCTGATGAGTTTCCTTCTATGTGTCCGGCAGAT 1020

1021 GGAAGAGTCCGTGGGATTACGCACAATAAAATATTGTGGGATTCATCCACTCTGGGGGCA 1080

1081 ATTCTGATGCGCAGAACTATTAGCAGTTGAGGGGGTAAAATGAAAAAAACATTATTAATA 1140

1141 GCTGCATCGCTTTCATTTTTTTCAGCAAGTGCGCTGGCGACGCCTGATTGTGTAACTGGA 1200

1201 AAGGTGGAGTATACAAAATATAATGATGACGATACCTTTACAGTTAAAGTGGGTGATAAA 1260

1261 GAATTATTTACCAACAGATGGAATCTTCAGTCTCTTCTTCTCAGTGCGCAAATTACGGGG 1320

1321 ATGACTGTAACCATTAAAACTAATGCCTGTCATAATGGAGGGGGATTCAGCGAAGTTATT 1380

1381 TTTCGTTGA 1389

FIG. 4

1 ATCGCATAGCTCATCGGAACAAGCTCAAGCGGTCTCCGGTCGAGTCCTCATGCGTCCATT 60

61 ATCTGCATTATGCGTTGTTAGCTCAGCCGGACAGAGCAATTGCCTTCTGAGCAATCGGTC 120

121 ACTGGTTCGAATCCAGTACAACGCGCCATATTTATTTACCAGGCTCGCTTTTGCGGGCCT 180

181 TTTTTATATCTGCGCCGGGTCTGGTGCTGATTACTTCAGCCAAAAGGAACACCTGTATAT 240

241 GAAGTGTATATTATTTAAATGGGTACTGTGCCTGTTACTGGGTTTTTCTTCGGTATCCTA 300

301 TTCCCGGGAGTTTACGATAGACTTTTCGACCCAACAAAGTTATGTCTCTTCGTTAAATAG 360

361 TATACGGACAGAGATATCGACCCCTCTTGAACATATATCTCAGGGGACCACATCGGTGTC 420

421 TGTTATTAACCACACCCCACCGGGCAGTTATTTTGCTGTGGATATACGAGGGCTTGATGT 480

481 CTATCAGGCGCGTTTTGACCATCTTCGTCTGATTATTGAGCAAAATAATTTATATGTGGC 540

541 CGGGTTCGTTAATACGGCAACAAATACTTTCTACCGTTTTTCAGATTTTACACATATATC 600

601 AGTGCCCGGTGTGACAACGGTTTCCATGACAACGGACAGCAGTTATACCACTCTGCAACG 660

661 TGTCGCAGCGCTGGAACGTTCCGGAATGCAAATCAGTCGTCACTCACTGGTTTCATCATA 720

721 TCTGGCGTTAATGGAGTTCAGTGGTAATACAATGACCAGAGATGCATCCAGAGCAGTTCT 780

781 GCGTTTTGTCACTGTCACAGCAGAAGCCTTACGCTTCAGGCAGATACAGAGAGAATTTCG 840

841 TCAGGCACTGTCTGAAACTGCTCCTGTGTATACGATGACGCCGGGAGACGTGGACCTCAC 900

901 TCTGAACTGGGGGCGAATCAGCAATGTGCTTCCGGAGTATCGGGGAGAGGATGGTGTCAG 960

961 AGTGGGGAGAATATCCTTTAATAATATATCAGCGATACTGGGGACTGTGGCCGTTATACT 1020

1021 GAATTGCCATCATCAGGGGGCGCGTTCTGTTCGCGCCGTGAATGAAGAGAGTCAACCAGA 1080

1081 ATGTCAGATAACTGGCGACAGGCCTGTTATAAAAATAAACAATACATTATGGGAAAGTAA 1140

1141 TACAGCTGCAGCGTTTCTGAACAGAAAGTCACAGTTTTTATATACAACGGGTAAATAAAG 1200

1201 GAGTTAAGCATGAAGAAGATGTTTATGGCGGTTTTATTTGCATTAGCTTCTGTTAATGCA 1260

1261 ATGGCGGCGGATTGTGCTAAAGGTAAAATTGAGTTTTCCAAGTATAATGAGGATGACACA 1320

1321 TTTACAGTGAAGGTTGACGGGAAAGAATACTGGACCAGTCGCTGGAATCTGCAACCGTTA 1380

1381 CTGCAAAGTGCTCAGTTGACAGGAATGACTGTCACAATCAAATCCAGTACCTGTGAATCA 1440

1441 GGCTCCGGATTTGCTGAAGTGCAGTTTAATAATGACTGA 1479

FIG. 5

1 gcggatccAA GGAATTTACC TTAGACTTCT CGACTGCAAA GACGTATGTA
51 GATTCGCTGA ATGTCATTCG CTCTGCAATA GGTACTCCAT TACAGACTAT
101 TTCATCAGGA GGTACGTCTT TACTGATGAT TGATAGTGGC TCAGGGGATA
151 ATTTGTTTGC AGTTGATGTC AGAGGGATAG ATCCAGAGGA AGGGCGGTTT
201 AATAATCTAC GGCTTATTGT TGAACGAAAT AATTTATATG TGACAGGATT
251 TGTTAACAGG ACAAATAATG TTTTTTATCG CTTTGCTGAT TTTTCACATG
301 TTACCTTTCC AGGTACAACA GCGGTTACAT TGTCTGGTGA CAGTAGCTAT
351 ACCACGTTAC AGCGTGTTGC AGGGATCAGT CGTACGGGGA TGCAGATAAA
401 TCGCCATTCG TTGACTACTT CTTATCTGGA TTTCCTGTCG CATAGTGGAA
451 CCTCACTGAC GCAGTCTGTG GCAAGAGCGA TGTTACGGTT TGTTACTGTG
501 ACAGCTGAAG CTTTACGTTT TCGGCAAATA CAGAGGGGAT TTCGTACAAC
551 ACTGGATGAT CTCAGTGGGC GTTCTTATGT AATGACTGCT GAAGATGTTG
601 ATGTTACATT GAACTGGGGA AGGTTGAGTA GCGTCCTGCC TGACTATCAT
651 GGACAAGACT CTGTTCGTGT AGGAAGAATT TCTTTTGGAA GCATTAATGC
701 AATTCTGGGA AGCGTGGCAT TAATACTGAA TTGTCATCAT CATGCATCGC
751 GAGTTGCCAG AATGGCATCT GATGAGTTTC CTTCTATGTG TCCGGCAGAT
801 GGAAGAGTCC GTGGGATTAC GCACAATAAA ATATTGTGGG ATTCATCCAC
851 TCTGGGGGCA ATTCTGATGC GCAGAACTAT ATATTGTGGG ATTCATCCAC
901 TGAAAAAAAC ATTATTAATA GCTGCATCGC TTTCATTTTT TTCAGCAAGT
951 GCGCTGGCGA CGCCTGATTG TGTAACTGGA AAGGTGGAGT ATACAAAATA
1001 TAATGATGAC GATACCTTTA CAGTTAAAGT GGGTGATAAA GAATTATTTA
1051 CCAACAGATG GAATCTTCAG TCTCTTCTTC TCAGTGCGCA AATTACGGGG
1101 ATGACTGTAA CCATTAAAAC TAATGCCTGT CATAATGGAG GGGGATTCAG
1151 CGAAGTTATT TTTCGTTGAC TCAGAATAGC TgCAGTGAAA AT

FIG. 6A

1 gcggatccga tgacgatgac aaaAAGGAAT TTACCTTAGA CTTCTCGACT
51 GCAAAGACGT ATGTAGATTC GCTGAATGTC ATTCGCTCTG CAATAGGTAC
101 TCCATTACAG ACTATTTCAT CAGGAGGTAC GTCTTTACTG ATGATTGATA
151 GTGGCTCAGG GGATAATTTG TTTGCAGTTG ATGTCAGAGG GATAGATCCA
201 GAGGAAGGGC GGTTTAATAA TCTACGGCTT ATTGTTGAAC GAAATAATTT
251 ATATGTGACA GGATTTGTTA ACAGGACAAA TAATGTTTTT TATCGCTTTG
301 CTGATTTTTC ACATGTTACC TTTCCAGGTA CAACAGCGGT TACATTGTCT
351 GGTGACAGTA GCTATACCAC GTTACAGCGT GTTGCAGGGA TCAGTCGTAC
401 GGGGATGCAG ATAAATCGCC ATTCGTTGAC TACTTCTTAT CTGGATTTAA
451 TGTCGCATAG TGGAACCTCA CTGACGCAGT CTGTGGCAAG AGCGATGTTA
501 CGGTTTGTTA CTGTGACAGC TGAAGCTTTA CGTTTTCGGC AAATACAGAG
551 GGGATTTCGT ACAACACTGG ATGATCTCAG TGGGCGTTCT TATGTAATGA
601 CTGCTGAAGA TGTTGATCTT ACATTGAACT GGGGAAGGTT GAGTAGCGTC
651 CTGCCTGACT ATCATGGACA AGACTCTGTT CGTGTAGGAA GAATTTCTTT
701 TGGAAGCATT AATGCAATTC TGGGAAGCGT GGCATTAATA CTGAATTGTC
751 ATCATCATGC ATCGCGAGTT GCCAGAATGG CATCTGATGA GTTTCCTTCT
801 ATGTGTCCGG CAGATGGAAG AGTCCGTGGG ATTACGCACA ATAAAATATT
851 GTGGGATTCA TCCACTCTGG GGGCAATTCT GATGCGCAGA ACTATTAGCA
901 GTTGAGGGGG TAAAATGAAA AAAACATTAT TAATAGCTGC ATCGCTTTCA
951 TTTTTTTCAG CAAGTGCGCT GGCGACGCCT GATTGTGTAA CTGGAAAGGT
1001 GGAGTATACA AAATATAATG ATGACGATAC CTTTACAGTT AAAGTGGGTG
1051 ATAAAGAATT ATTTACCAAC AGATGGAATC TTCAGTCTCT TCTTCTCAGT
1101 GCGCAAATTA CGGGGATGAC TGTAACCATT AAAACTAATG CCTGTCATAA
1151 TGGAGGGGGA TTCAGCGAAG TTATTTTTCG TTGACTCAGA ATAGCTgCAG
1201 TGAAAAT

FIG. 6B

1 gcatatgcat caccatcacc atcacAAGGA ATTTACCTTA GACTTCTCGA
51 CTGCAAAGAC GTATGTAGAT TCGCTGAATG TCATTCGCTC TGCAATAGGT
101 ACTCCATTAC AGACTATTTC ATCAGGAGGT ACGTCTTTAC TGATGATTGA
151 TAGTGGCTCA GGGGATAATT TGTTTGCAGT TGATGTCAGA GGGATAGATC
201 CAGAGGAAGG GCGGTTTAAT AATCTACGGC TTATTGTTGA ACGAAATAAT
251 TTATATGTGA CAGGATTTGT TAACAGGACA AATAATGTTT TTTATCGCTT
301 TGCTGATTTT TCACATGTTA CCTTTCCAGG TACAACAGCG GTTACATTGT
351 CTGGTGACAG TAGCTATACC ACGTTACAGC GTGTTGCAGG GATCAGTCGT
401 ACGGGGATGC AGATAAATCG CCATTCGTTG ACTACTTCTT ATCTGGATTT
451 AATGTCGCAT AGTGGAACCT CACTGACGCA GTCTGTGGCA AGAGCGATGT
501 TACGGTTTGT TACTGTGACA GCTGAAGCTT TACGTTTTCG GCAAATACAG
551 AGGGGATTTC GTACAACACT GGATGATCTC AGTGGGCGTT CTTATGTAAT
601 GACTGCTGAA GATGTTGATC TTACATTGAA CTGGGGAAGG TTGAGTAGCG
651 TCCTGCCTGA CTATCATGGA CAAGACTCTG TTCGTGTAGG AAGAATTTCT
701 TTTGGAAGCA TTAATGCAAT TCTGGGAAGC GTGGCATTAA TACTGAATTG
751 TCATCATCAT GCATCGCGAG TTGCCAGAAT GGCATCTGAT GAGTTTCCTT
801 CTATGTGTCC GGCAGATGGA AGAGTCCGTG GGATTACGCA CAATAAAATA
851 TTGTGGGATT CATCCACTCT GGGGGCAATT CTGATGCGCA GAACTATTAG
901 CAGTTGAGGG GGTAAAATGA AAAAAACATT ATTAATAGCT GCATCGCTTT
951 CATTTTTTTC AGCAAGTGCG CTGGCGACGC CTGATTGTGT AACTGGAAAG
1001 GTGGAGTATA CAAAATATAA AGATGACGAT ACCTTTACAG TTAAAGTGGG
1051 TGATAAAGAA TTATTTACCA ACAGATGGAA TCTTCAGTCT CTTCTTCTCA
1101 GTGCGCAAAT TACGGGGATG ACTGTAACCA TTAAAACTAA TGCCTGTCAT
1151 AATGGAGGGG GATTCAGCGA AGTTATTTTT CGTTGACTCA GAATAGCTCA
1201 GTGAAAATAG CAGGCGGAGA TaTCgATAAA TGTTA

FIG. 6C

1 gcggatcCGG GAGTTTACGA TAGACTTTTC GACCCAACAA AGTTATGTCT
51 CTTCGTTAAA TAGTATACGG ACAGAGATAT CGACCCCTCT TGAACATATA
101 TCTCAGGGGA CCACATCGGT GTCTGTTATT AACCACACCC CACCGGGCAG
151 TTATTTTGCT GTGGATATAC GAGGGCTTGA TGTCTATCAG GCGCGTTTTG
201 ACCATCTTCG TCTGATTATT GAGCAAAATA ATTTATATGT GGCCGGGTTC
251 GTTAATACGG CAACAAATAC TTTCTACCGT TTTTCAGATT TTACACATAT
301 ATCAGTGCCC GGTGTGACAA CGGTTTCCAT GACAACGGAC AGCAGTTATA
351 CCACTCTGCA ACGTGTCGCA GCGCTGGAAC GTTCCGGAAT GCAAATCAGT
401 CGTCACTCAC TGGTTTCATC ATATCTGGCG TTAATGGAGT TCAGTGGTAA
451 TACAATGACC AGAGATGCAT CCAGAGCAGT TCTGCGTTTT GTCACTGTCA
501 CAGCAGAAGC CTTACGCTTC AGGCAGATAC AGAGAGAATT TCGTCAGGCA
551 CTGTCTGAAA CTGCTCCTGT GTATACGATG ACGCCGGGAG ACGTGGACCT
601 CACTCTGAAC TGGGGGCGAA TCAGCAATGT GCTTCCGGAG TATCGGGGAG
651 AGGATGGTGT CAGAGTGGGG AGAATATCCT TTAATAATAT ATCAGCGATA
701 CTGGGGACTG TGGCCGTTAT ACTGAATTGC CATCATCAGG GGGCGCGTTC
751 TGTTCGCGCC GTGAATGAAG AGAGTCAACC AGAATGTCAG ATAACTGGCG
801 ACAGGCCTGT TATAAAAATA AACAATACAT TATGGGAAAG TAATACAGCT
851 GCAGCGTTTC TGAACAGAAA GTCACAGTTT TTATATACAA CGGGTAAATA
901 AAGGAGTTAA GCATGAAGAA GATGTTTATG GCGGTTTTAT TTGCATTAGC
951 TTCTGTTAAT GCAATGGCGG CGGATTGTGC TAAAGGTAAA ATTGAGTTTT
1001 CCAAGTATAA TGAGGATGAC ACATTTACAG TGAAGGTTGA CGGGAAAGAA
1051 TACTGGACCA GTCGCTGGAA TCTGCAACCG TTACTGCAAA GTGCTCAGTT
1101 GACAGGAATG ACTGTCACAA TCAAATCCAG TACCTGTGAA TCAGGCTCCG
1151 GATTTGCTGA AGTGCAGTTT AATAATGACT GAGGCATAAg CTtATTCGTG
1201 G

FIG. 7A

```
1    gcggatccga tgacgatgac aaaCGGGAGT TTACGATAGA CTTTTCGACC
51   CAACAAAGTT ATGTCTCTTC GTTAAATAGT ATACGGACAG AGATATCGAC
101  CCCTCTTGAA CATATATCTC AGGGGACCAC ATCGGTGTCT GTTATTAACC
151  ACACCCCACC GGGCAGTTAT TTTGCTGTGG ATATACGAGG GCTTGATGTC
201  TATCAGGCGC GTTTTGACCA TCTTCGTCTG ATTATTGAGC AAAATAATTT
251  ATATGTGGCC GGGTTCGTTA ATACGGCAAC AAATACTTTC TACCGTTTTT
301  CAGATTTTAC ACATATATCA GTGCCCGGTG TGACAACGGT TTCCATGACA
351  ACGGACAGCA GTTATACCAC TCTGCAACGT GTCGCAGCGC TGGAACGTTC
401  CGGAATGCAA ATCAGTCGTC ACTCACTGGT TTCATCATAT CTGGCGTTAA
451  TGGAGTTCAG TGGTAATACA ATGACCAGAG ATGCATCCAG AGCAGTTCTG
501  CGTTTTGTCA CTGTCACAGC AGAAGCCTTA CGCTTCAGGC AGATACAGAG
551  AGAATTTCGT CAGGCACTGT CTGAAACTGC TCCTGTGTAT ACGATGACGC
601  CGGGAGACGT GGACCTCACT CTGAACTGGG GGCGAATCAG CAATGTGCTT
651  CCGGAGTATC GGGGAGAGGA TGGTGTCAGA GTGGGGAGAA TATCCTTTAA
701  TAATATATCA GCGATACTGG GGACTGTGGC CGTTATACTG AATTGCCATC
751  ATCAGGGGGC GCGTTCTGTT CGCGCCGTGA ATGAAGAGAG TCAACCAGAA
801  TGTCAGATAA CTGGCGACAG GCCTGTTATA AAAATAAACA ATACATTATG
851  GGAAAGTAAT ACAGCTGCAG CGTTTCTGAA CAGAAAGTCA CAGTTTTTAT
901  ATACAACGGG TAAATAAAGG AGTTAAGCAT GAAGAAGATG TTTATGGCGG
951  TTTTATTTGC ATTAGCTTCT GTTAATGCAA TGGCGGCGGA TTGTGCTAAA
1001 GGTAAAATTG AGTTTTCCAA GTATAATGAG GATGACACAT TTACAGTGAA
1051 GGTTGACGGG AAAGAATACT GGACCAGTCG CTGGAATCTG CAACCGTTAC
1101 TGCAAAGTGC TCAGTTGACA GGAATGACTG TCACAATCAA ATCCAGTACC
1151 TGTGAATCAG GCTCCGGATT TGCTGAAGTG CAGTTTAATA ATGACTGAGG
1201 CATAAgCTtA TTCGTGG
```

FIG. 7B

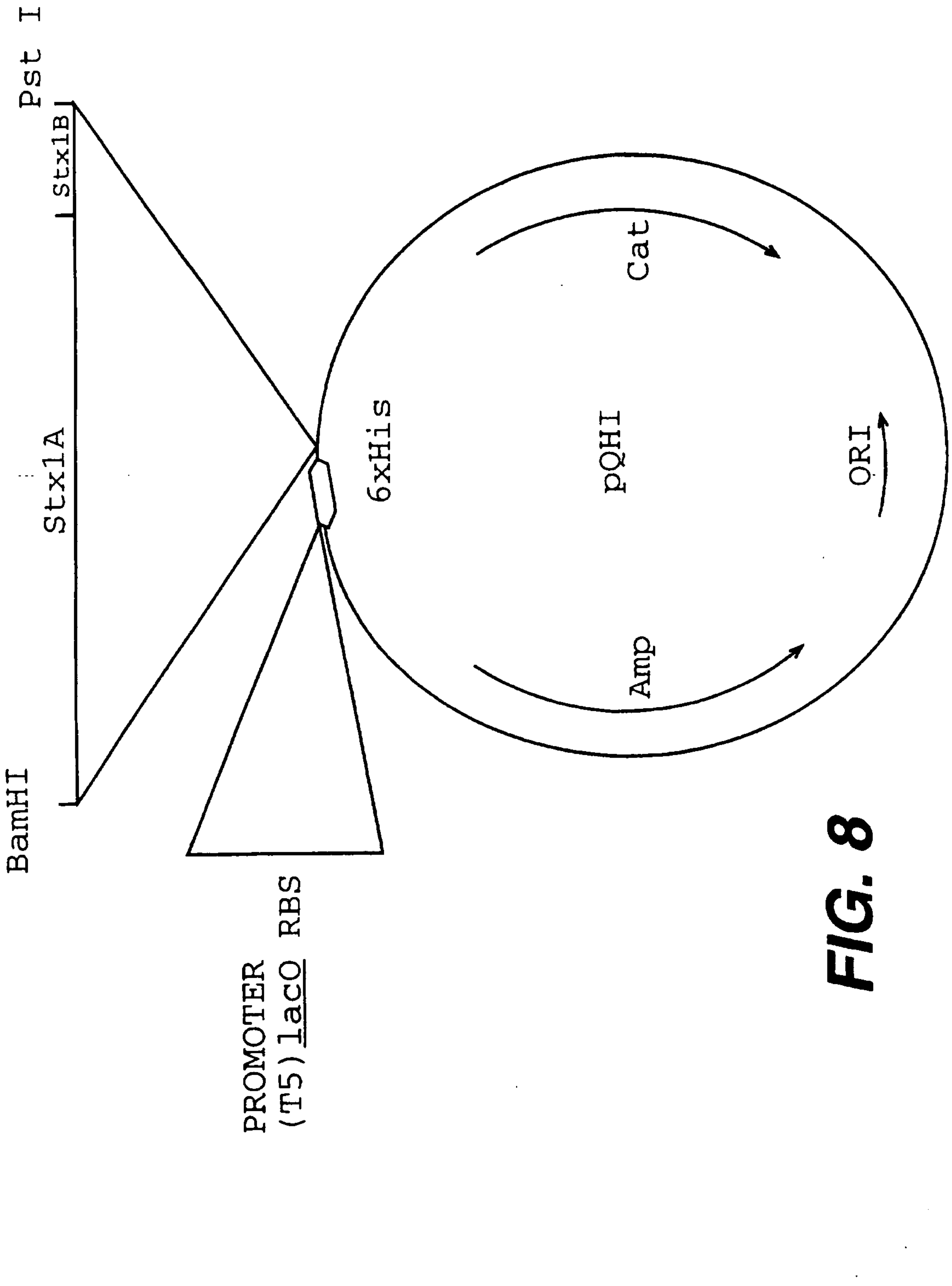

Pst I
Stx1B
Stx1A
BamHI
PROMOTER
(T5) lacO RBS
6xHis
pQHI
Cat
ORI
Amp

FIG. 8

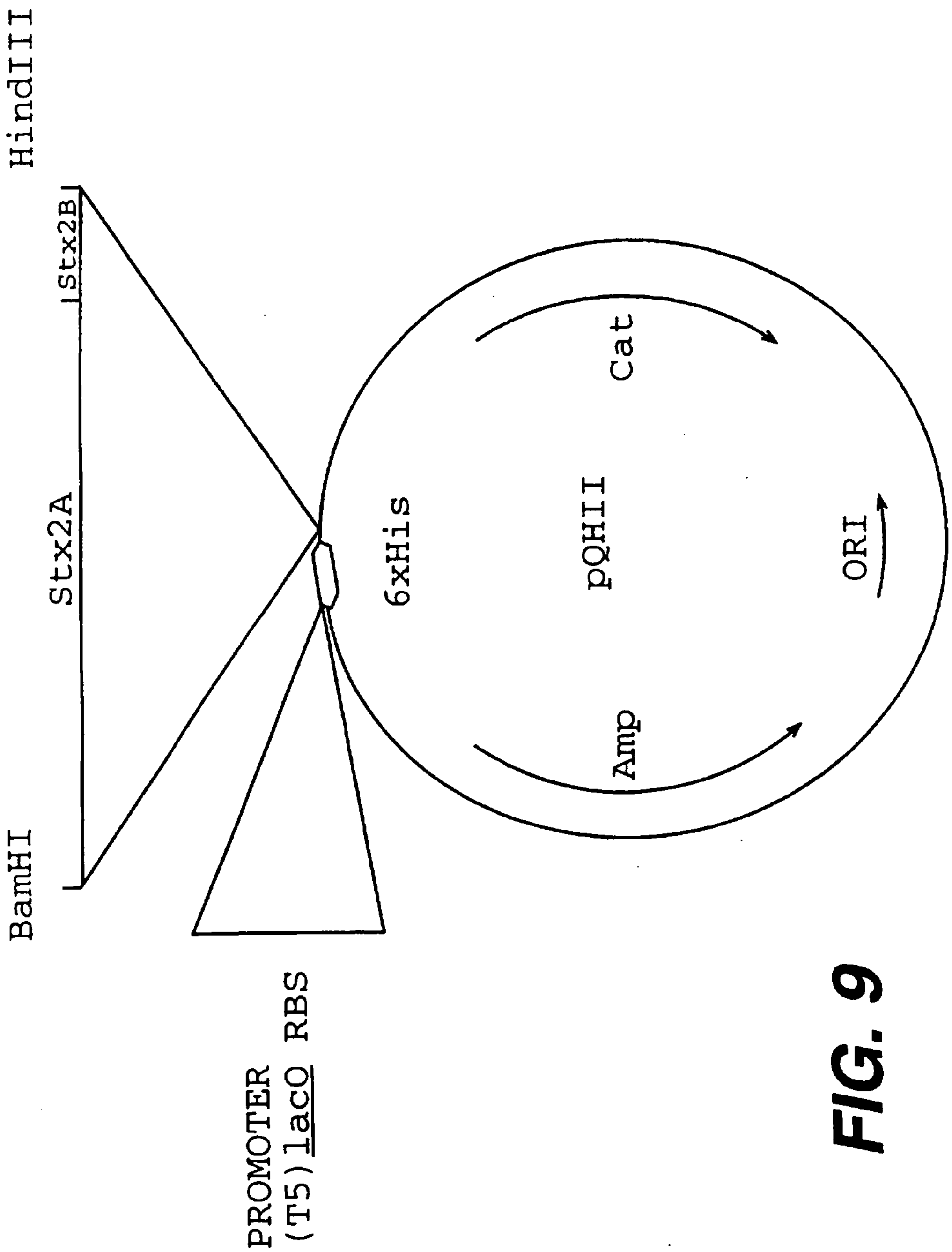

HindIII
Stx2B
Stx2A
BamHI
Cat
6xHis
pQHII
ORI
Amp
PROMOTER
(T5) lacO RBS

FIG. 9

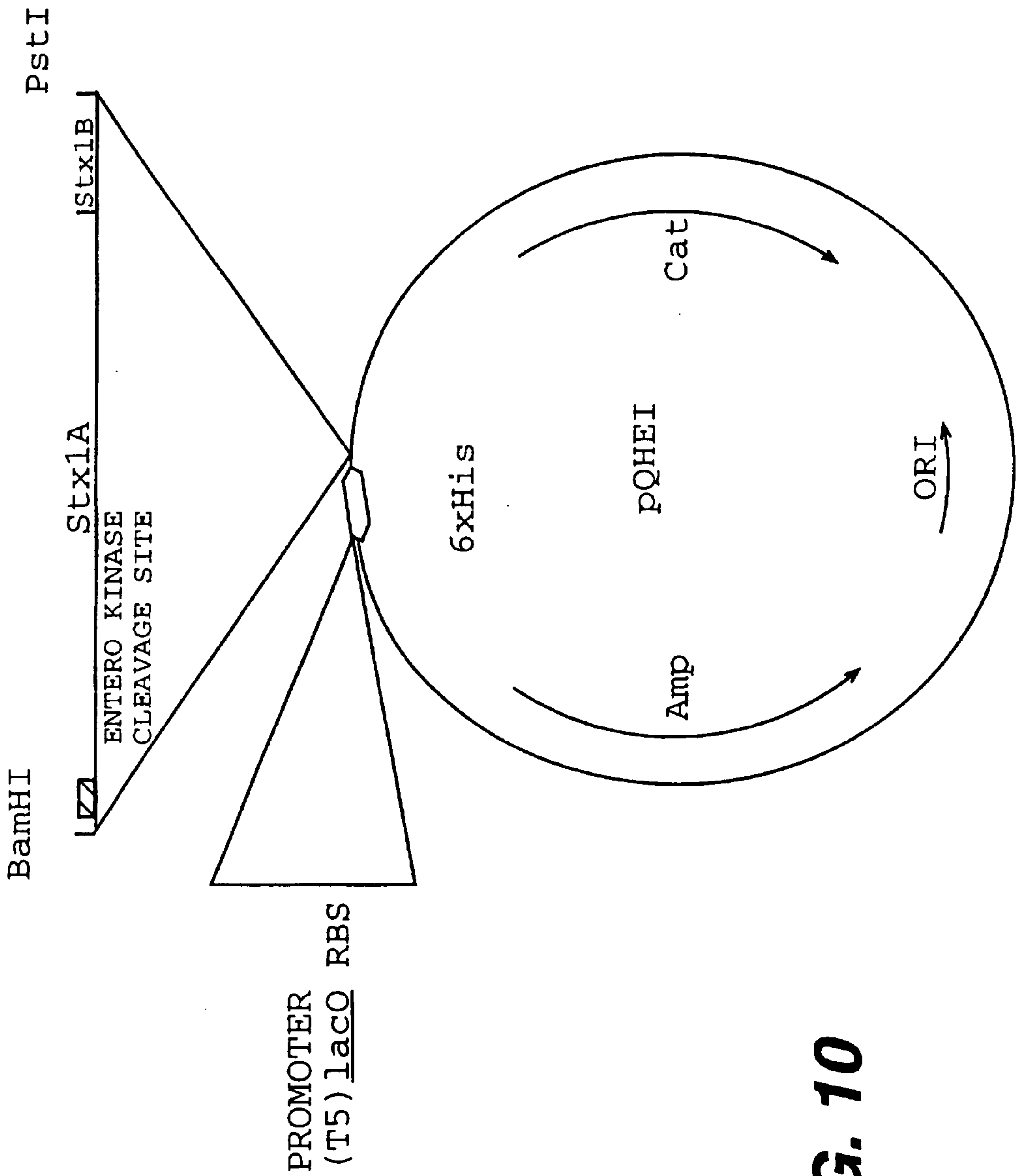

PstI
Stx1B
Stx1A
ENTERO KINASE
CLEAVAGE SITE
BamHI
PROMOTER
(T5) lacO RBS
6xHis
pQHEI
Cat
ORI
Amp

FIG. 10

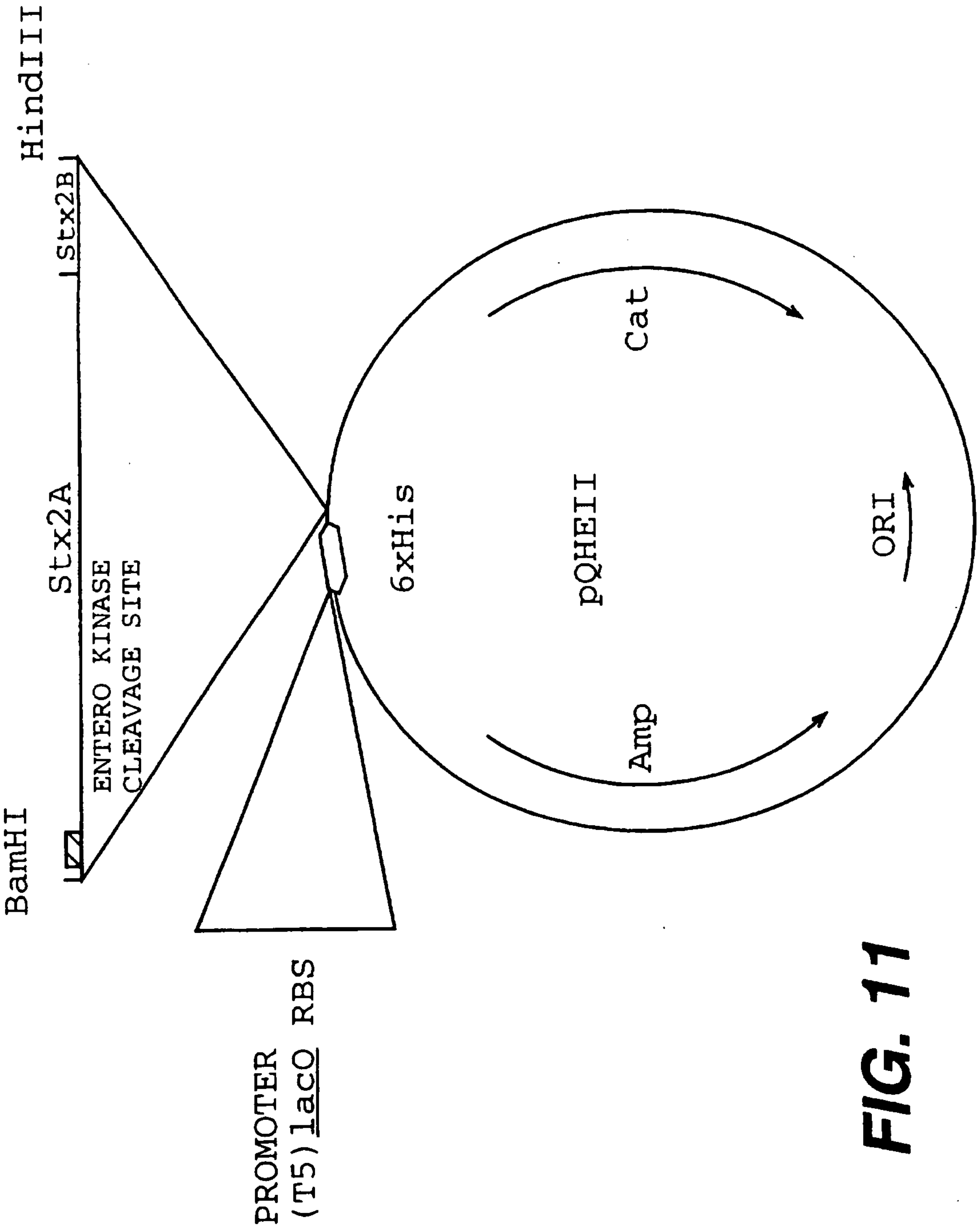

HindIII
Stx2B
Stx2A
ENTERO KINASE
CLEAVAGE SITE
BamHI
PROMOTER
(T5) lacO RBS
6xHis
pQHEII
Cat
ORI
Amp

FIG. 11

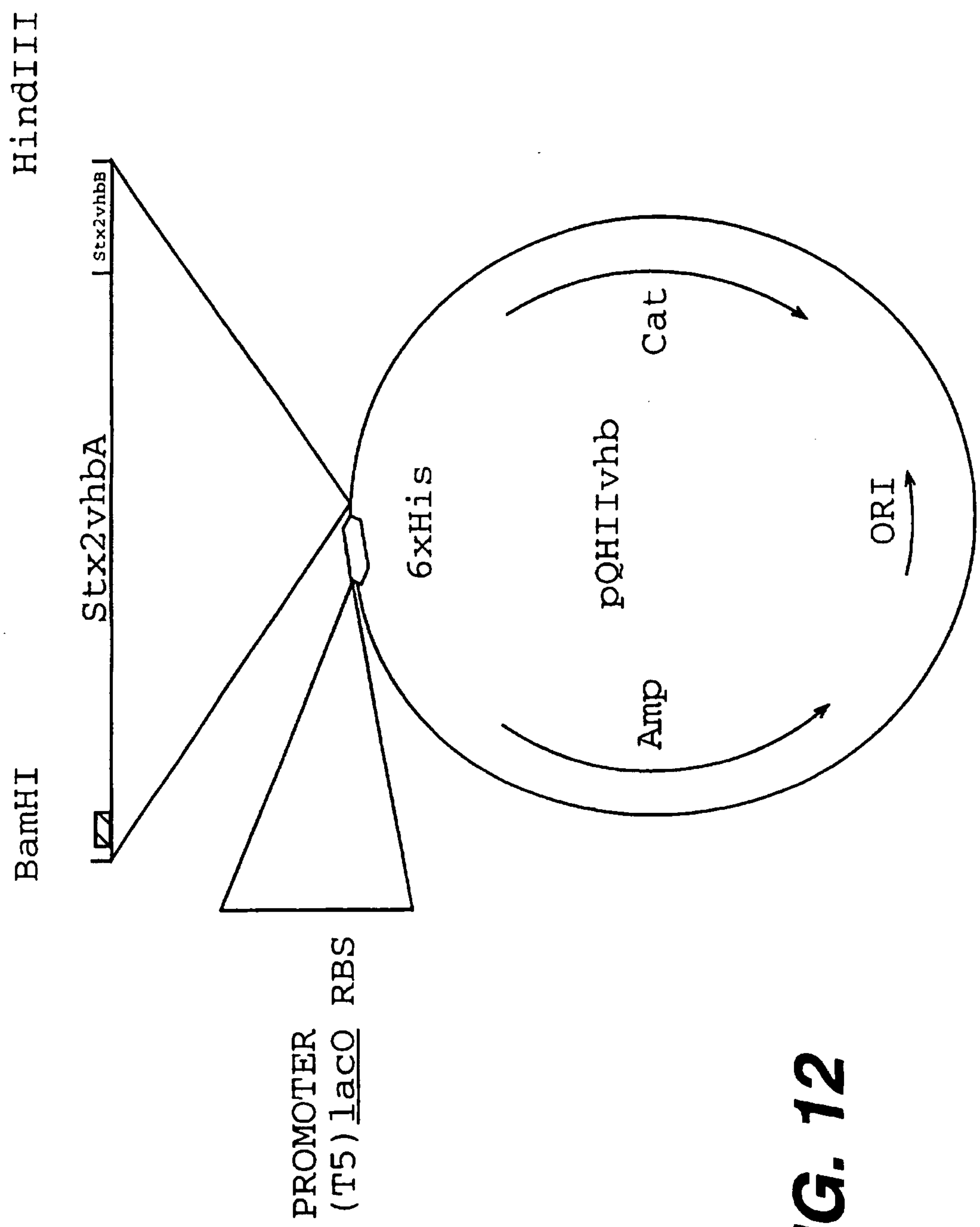

HindIII
Stx2vhbB
Stx2vhbA
BamHI
6xHis
pQHIIvhb
Cat
ORI
Amp
PROMOTER
(T5) lacO RBS

FIG. 12

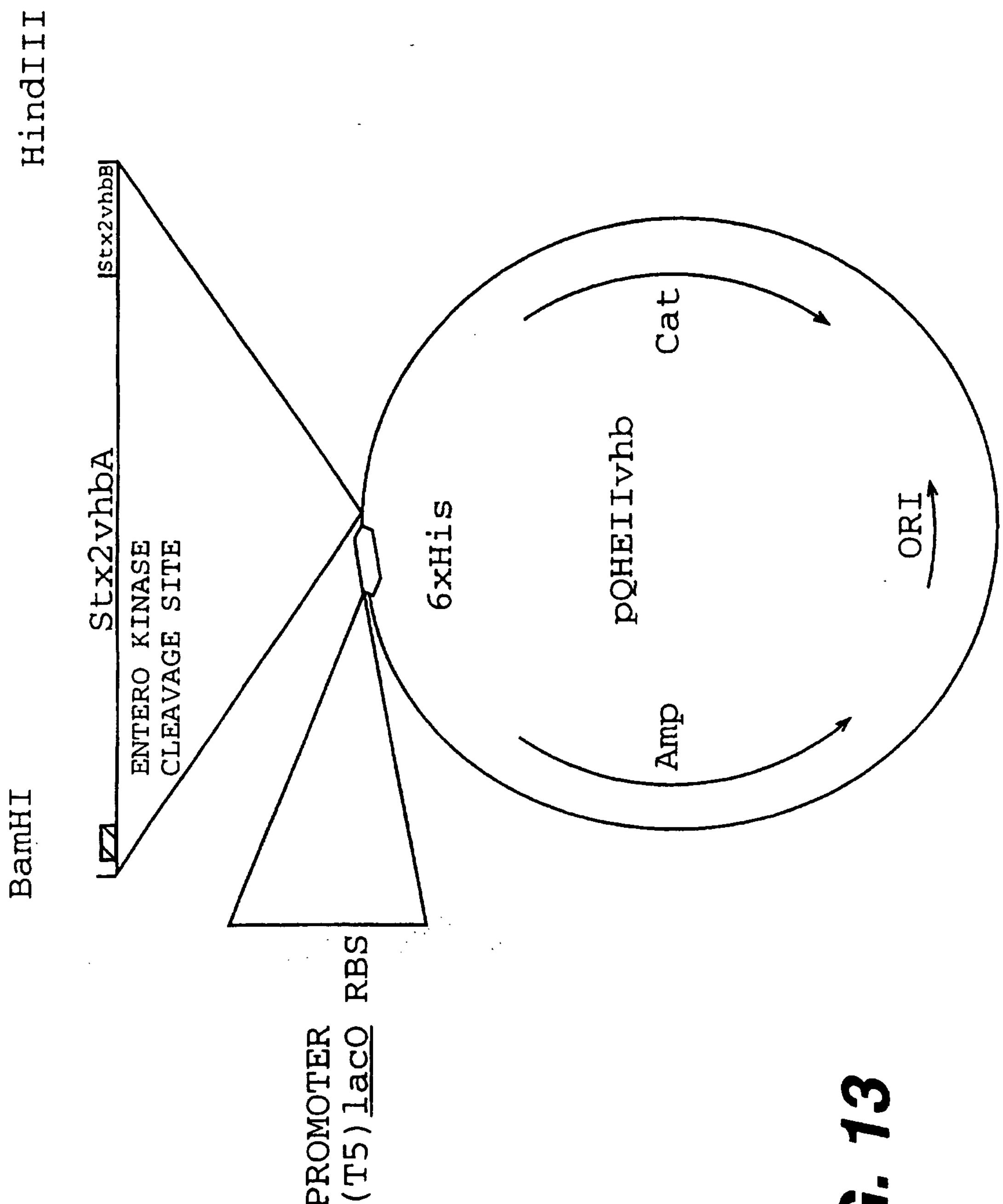

HindIII
Stx2vhbB
Stx2vhbA
ENTERO KINASE
CLEAVAGE SITE
BamHI
Cat
pQHEIIvhb
6xHis
ORI
Amp
PROMOTER
(T5) lacO RBS

FIG. 13

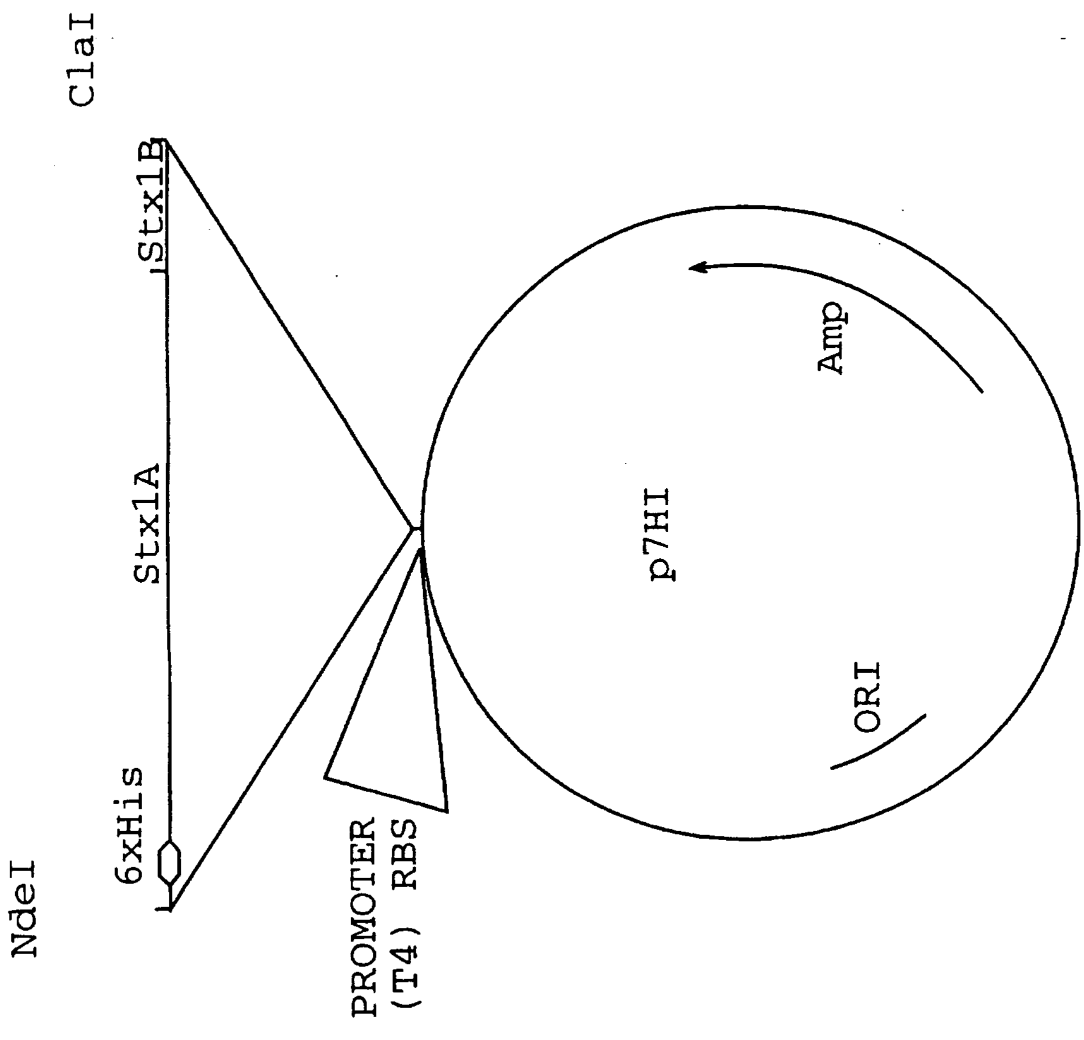


FIG. 14

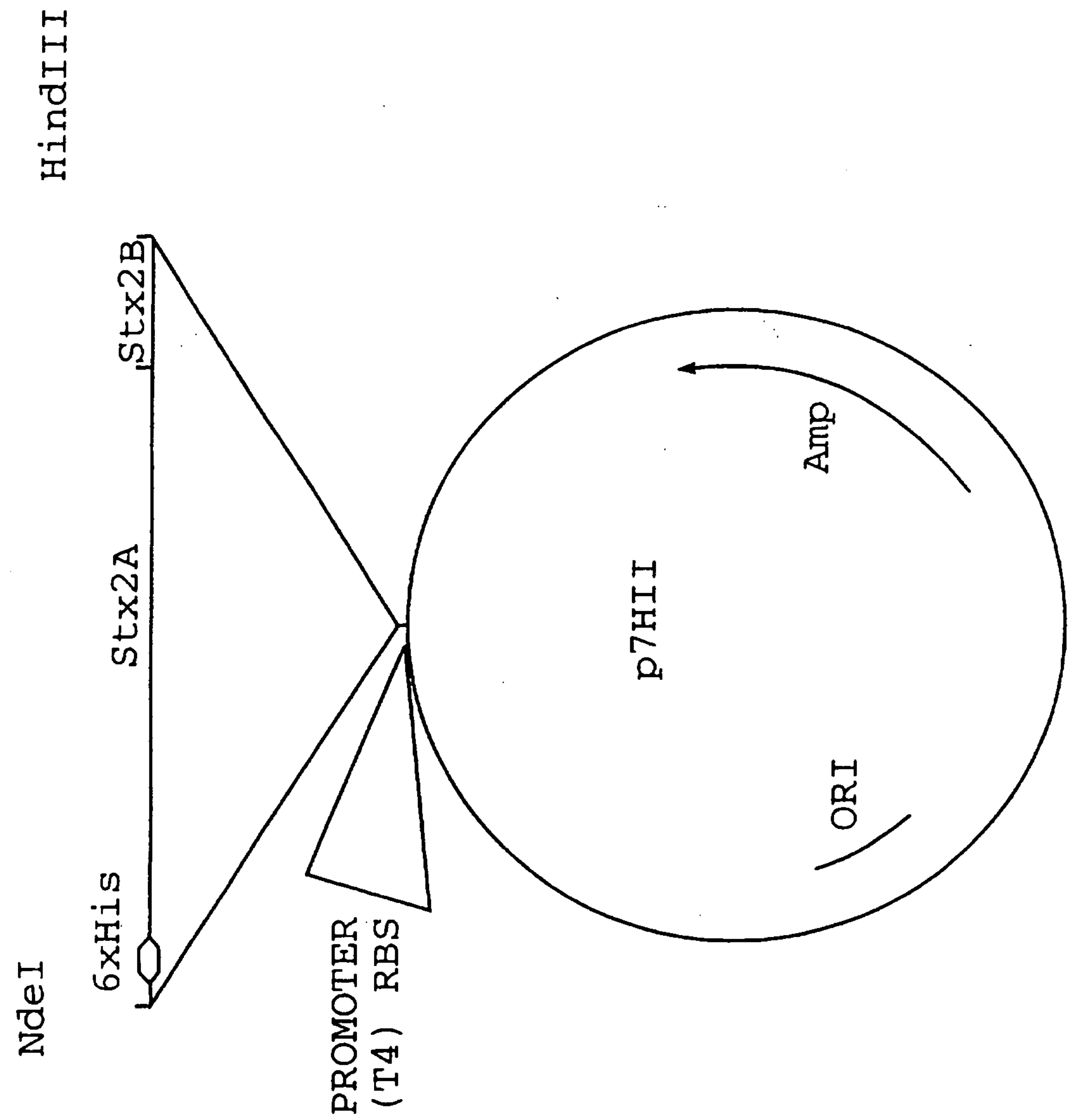

HindIII
Stx2B
Stx2A
6xHis
NdeI
PROMOTER
(T4) RBS
Amp
p7HII
ORI

FIG. 15

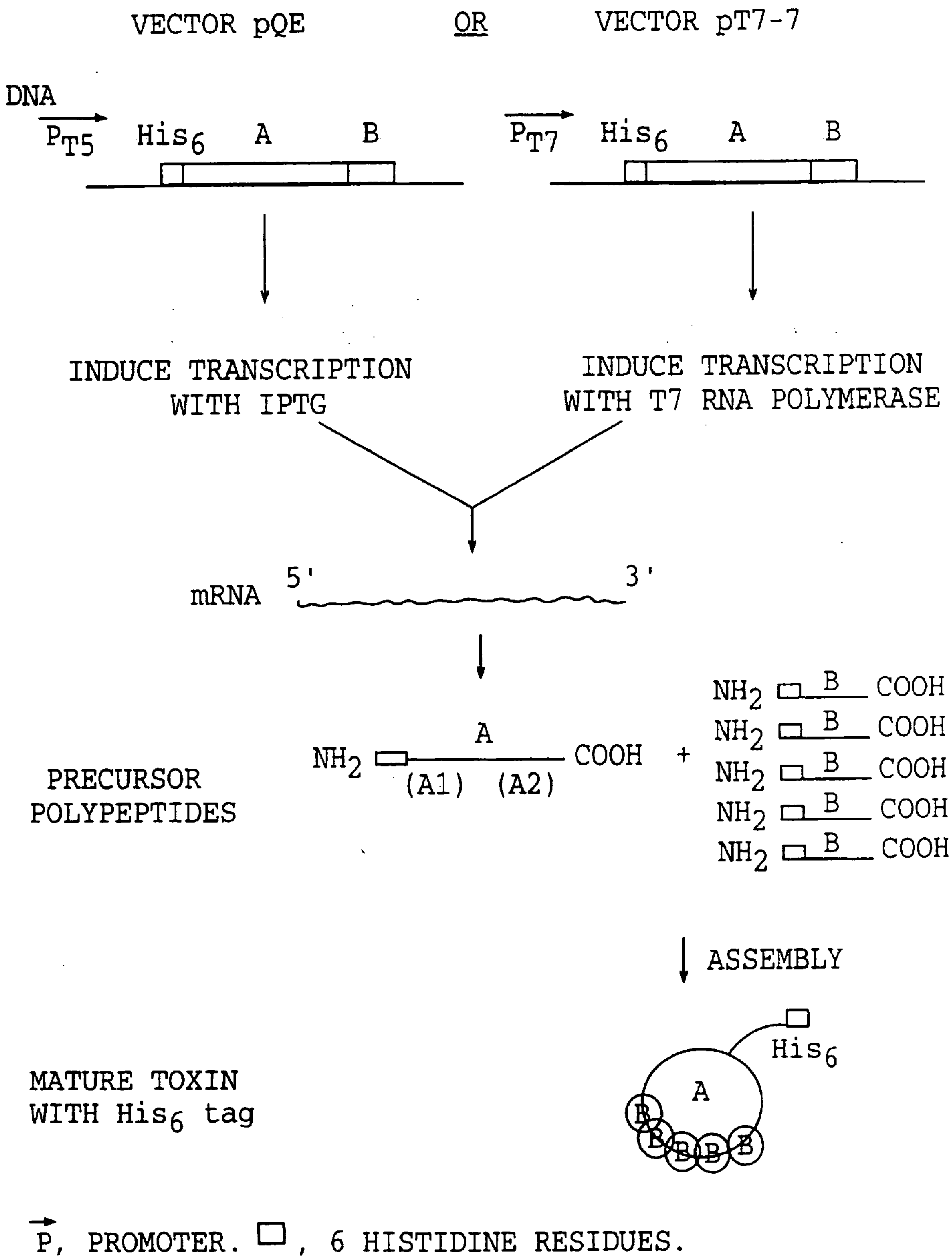

VECTOR pQE
OR
VECTOR pT7-7
DNA
P_{T5} His_6 A B
P_{T7} His_6 A B
INDUCE TRANSCRIPTION WITH IPTG
INDUCE TRANSCRIPTION WITH T7 RNA POLYMERASE
mRNA 5' 3'
PRECURSOR POLYPEPTIDES
NH_2 A COOH
(A1) (A2)
+
NH_2 B COOH
NH_2 B COOH
NH_2 B COOH
NH_2 B COOH
NH_2 B COOH
ASSEMBLY
MATURE TOXIN WITH His_6 tag
His_6
A
B B B B B
P, PROMOTER. □, 6 HISTIDINE RESIDUES.

FIG. 16